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FOREWORD

THE splitting of scientific disciplines cannot be welcome for its own sake ; but, like the natural break-up of families when adolescence is past, it is a normal sequel to maturity. First-class work on the physiology of insects was being published more than a century ago ; but the speed of development in this field has increased immensely in very recent years ; there can be no doubt that the time has come when that development will be encouraged by the provision of a separate medium for publication.

It would be a misfortune if the study of insect physiology were to be divorced from other branches of biology, for a general physiology, a universal biology, is the objective of us all. If it is to have its full value, physiology must be comparative. The Journal which makes such an auspicious début with the present number will be concerned not only with the physiology of insects, but with the physiology of other groups of arthropods, particularly of terrestrial forms. The fact that original work on biochemistry, toxicology, and the functional aspects of morphology will be published ensures a catholic approach.

It is the declared policy of the Journal that preference will be given to papers that contribute to an understanding of the general principles underlying the physiology of insects. These principles will not be found to differ from those underlying the physiology of other organisms. It is therefore to be hoped that, just as the insect physiologist must keep himself fully informed about the physiology of other groups of animals, so the mammalian physiologist may

come to realize that the key to many of the fundamental problems of physiology may perhaps be found most readily among the insects.

But, in order to utilize this wonderful biological material to full advantage, knowledge is necessary. It is the aim of the JOURNAL OF INSECT PHYSIOLOGY to foster the development of that knowledge by attracting to one focus a selection of the most important new contributions from all parts of the world. The enterprise will have the goodwill of the rapidly growing company of insect physiologists everywhere.

V. B. WIGGLESWORTH

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THE REGULATION OF TASTE THRESHOLDS FOR SUGARS IN THE BLOWFLY*

D. R. EVANS† and V. G. DETHIER

Department of Biology, The Johns Hopkins University, Baltimore 18, Md.

(*Received* 26 *November* 1956)

Abstract—The feeding of glucose, fucose, mannose, and lactose to *Phormia* elevates its tarsal thresholds to glucose, fucose, fructose, and sucrose even though two of the fed sugars are tasteless (mannose and lactose) and two are completely nonnutritious (lactose and fucose). No action of sugars on external chemoreceptors can account for a prolonged threshold elevation. The time course of blood-sugar changes after feeding cannot be related to that of taste threshold change. Injection of several sugars into the blood does not affect sugar thresholds. Ligation of the crop duct before or after feeding sugar solutions does not prevent elevation of sugar thresholds. The period of elevation is shortened, however, as a result of the smaller quantities ingested. By elimination of other possibilities, it is concluded that the locus of sugar action in elevating sugar threshold is some region of the gut other than the crop. The normal blood sugar of adult *Phormia* is trehalose, which has been found in concentrations from 1·25 to 30 g/l. Larval stages appear to lack this sugar or else contain it in very low concentrations. Small concentrations of glucose have consistently been found in both larval and adult blood. Flight rapidly decreases blood trehalose to a small fraction of its normal value. The taste thresholds of the honeybee are altered by feeding, starvation, and flight in a manner similar to the alterations in *Phormia*.

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In an earlier communication it was suggested that much of the feeding behaviour of the blowfly *Phormia regina* could be explained in terms of taste thresholds (Dethier *et al.*, 1956). The measure of taste thresholds which we have employed is proboscis extension, the first act of the feeding behaviour of the blowfly (Dethier and Chadwick, 1947, 1948a). If, after proboscis extension, the labellar hairs encounter an acceptable substance during the exploratory probings that occur, the labellar lobes open and sucking commences. The rate of sucking depends largely on the "sweetness" of the solution, although viscosity plays a hindering rôle at very high concentrations. Again threshold is involved, since the "sweetness" of a given solution depends on the sensitivity of the organism at the time to that substance. Feeding apparently continues as long as there is a continuous sensory input from the sugar-sensitive receptors, but intake of a solution normally stops far short of the maximum crop capacity. Adaptation of the contact chemoreceptors involved seemingly removes the sensory input necessary to maintain sucking, and feeding is terminated. The time required for adaptation to occur is related to the stimulating effectiveness of the solution and the particular sugar involved. Normally then, proboscis extension, the rate of sucking, and the duration of feeding all depend in part on the threshold of the organism at that moment for the substance encountered.

* This work was aided by a grant from the National Science Foundation.

† National Science Foundation Fellow.

Thresholds of the contact chemoreceptors in insects are known to be altered by a number of factors. Dethier and Chadwick (1948a), reviewing the subject, listed the following factors as affecting gustatory thresholds: conditioning, age, preparation of specimens, temperature, humidity, sex, odours, preceding chemical stimulation (state of adaptation, substances augmenting or inhibiting the response of the sugar receptor, substances which are rejected and centrally inhibit the sugar receptor, etc.), and the nutritional state of the animal. The present account will be concerned almost exclusively with the last factor, nutritional state (or more generally the various consequences of feeding and starvation), since primarily this factor would be expected to be responsible for the obvious ability of animals to respond adaptively to food. That the blowfly does not use nutritional adequacy as the criterion of acceptable food has been clearly demonstrated, although the kind and amount of a sugar which will be ingested and the effect of this ingestion on subsequent responses to food is highly selective (Dethier *et al.*, 1956).

Sugars were chosen for this study of ingestion and chemosensory response since the response of the contact chemoreceptors has been more thoroughly studied with respect to sugars than to other nutritious substances, and every possible combination of taste response and nutritional value is easily obtained with sugars.

Earlier work concerning the effect of starvation on taste thresholds of insects is somewhat contradictory. Though we shall be primarily concerned here with responses to sugars, it should be noted that the change of threshold upon starvation is not necessarily the same for all substances. Even the various acceptable sugars have been reported to have different sensitivity curves during starvation (Haslinger, 1935). Most investigators found an increased sensitivity to sugars during a period of starvation (see Dethier and Chadwick, 1948a, for a review), but a number of others reported no change or even an increased threshold (an increased sugar threshold is regularly observed in *Phormia* shortly before death). Von Frisch (1934) compared the sucrose threshold of bees on a sugar-water diet just adequate to maintain life with that of bees which had access to concentrated sugar water and a hive full of honey. Both groups had a sucrose acceptance threshold between 0·5 and 1·0 M. But a distinctly lower sucrose threshold was found when the natural food was scarce than when it was abundant. Kunze (1927) made a similar observation. Elsewhere von Frisch (1950) has stated that a starvation period of several hours regularly suffices to lower the sugar-acceptance threshold of the bee to sugar to a value of 1/8 to 1/16 M. The nutritional state of the animals at the start of the starvation period was, however, unknown. Since the bee seems somewhat anomalous in this regard, we have run a few experiments on bees to check this point (see below).

Minnich found that starvation markedly increased sugar sensitivity of the tarsal chemoreceptors of the butterfly *Pyrameis* (1922) and the blowfly *Calliphora* (1929), but did not alter the threshold of the labellar receptors of *Calliphora* (1931).

MATERIALS AND METHODS

This study is based upon measurements of threshold, crop content, and blood-sugar levels under a variety of experimental conditions. Unless otherwise specified,

sugar thresholds are tarsal thresholds obtained by the methods of DETHIER and CHADWICK (1947, 1948b). All flies had emerged 1 to 3 days before the start of the experiment.

Crop weight and crop-sugar determinations were performed on the crops of individual flies at various intervals after feeding. Since the crop of *Phormia* is a tough-walled, blind sac at the end of a narrow duct from the proventriculus, it is easily removed without loss of its contents. Weights were obtained immediately after removal on a ROLLER-SMITH balance (sensitivity *c.* 0·05 mg). Sugar determinations were run on the same crops after they had been crushed in 5% trichloroacetic acid.

With some difficulty it proved feasible to ligate the crop duct either before or after feeding. The duct occupies a superficial ventral position between the abdomen and thorax. The incision was covered with vaseline after the ligation. It is probable that the ligature constricts the crop nerve, since in *Drosophila* the nerve follows the duct (DEMEREC, 1950); no attempt was made to confirm this for *Phormia*. Mortality was fairly high, but some flies lived more than 24 hours after the operation.

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Solutions of sugars were injected into the haemocoel of the flies with the micro-injection apparatus of BUCK (1949). The fine glass needles were inserted into the thorax through the membrane between the abdomen and thorax just to one side of the mid-ventral line. The amount of solution delivered by a given fractional turn of the drive shaft was determined directly by measuring the quantity of sugar delivered into a volume of water when the syringe was filled with the concentration of sugar to be injected.

The anthrone method of DIMLER *et al.* (1952) proved convenient and reliable for the quantitative determination of both reducing and nonreducing carbohydrates. For small amounts of reducing sugars the micro-ferricyanide method of PARK and JOHNSON (1949) was very reliable. Because of wide deviation from linearity, determinations were not made on samples containing more than 10μg (as glucose). In a few experiments the DISCHE (1956) method was employed for estimating fucose in the presence of hexoses.

Samples of blood, unless otherwise specified, were samples of whole blood collected from live flies. Measured volumes of blood (1–4μl per fly) were withdrawn from the base of the amputated forelegs of adults into a graduated micropipette, made from thermometer tubing. Mere puncture of the body wall of turgid larvae produced a large drop of blood (up to 20μl). Linearity of delivery of the pipette was demonstrated by the method of BUCK *et al.* (1950); absolute delivery volume was determined by titrating a delivered volume of sulphuric acid with standard NaOH (KIRK, 1950).

The identification and quantitative determination of blood sugars was accomplished by paper chromatographic separation, chromatographic elution, and estimation of carbohydrates in the eluate by the above-mentioned sugar methods. A row of 2μl samples was applied to the baseline of the paper, the number depending upon the quantity of sugar expected. The paper rested on a warming

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pan at 50° C so that drying was nearly immediate. Of the variety of systems tested, continuous ascending development (FISCHBACH and LEVINE, 1955) employing *n*-propanol-ethyl acetate-water (7 : 1 : 1 v/v) (BARR, 1954) or *n*-butanol-ethanol-acetone-water (5 : 4 : 3 : 2 v/v) (GRAY and FRAENKEL, 1954) as solvents was found to give the best separations. Location of reducing spots on the chromatograms was accomplished by the ammoniacal silver method of TREVELYAN *et al.* (1950). For the detection of small amounts of trehalose and other nonreducing carbohydrates, the published methods utilizing periodate to convert *vic*-glycols to aldehydes (LEMIEUX and BAUER, 1954; and CIFONELLI and SMITH, 1954) proved unreliable in our hands. However, spraying with 0·5% sodium *meta* periodate in water and drying at 20 to 60° C followed by the silver method of TREVELYAN *et al.* (1950) proved sensitive and reproducible. In all cases the coloured chromatograms were made permanent by being treated for a few minutes in Kodak Acid Fixer followed by a water wash.

The elution method of DIMLER *et al.* (1952) allowed recovery of the sugars in a small volume of water which could be rapidly evaporated to dryness *in vacuo*.

Acid hydrolysis was performed on small quantities of unknown carbohydrates from chromatograms in order to identify the products by rechromatography. This procedure was readily accomplished by the neat micro method of PORTER and HOBAN (1954).

An important result of the application of these methods to the blood of *Phormia* was that completely satisfactory separations of blood sugars of whole blood were obtained provided that samples of 2μl or less were applied at a single spot on the baseline. This finding permitted the investigation of carbohydrates in blood samples which would have been impracticably small had the usual desalting and purification procedures always been necessary to achieve reliable separation of sugars.

RESULTS AND DISCUSSION

Effect of feeding on threshold

The effect of feeding on the sugar thresholds of *Phormia* was investigated by feeding the flies with four sugars: glucose and fucose, which are highly stimulating; mannose, which is weakly stimulating; and lactose, which is nonstimulating. Glucose and mannose support life well; lactose is the same as or insignificantly better than water alone; and fucose is the same as water alone or is perhaps even toxic (HASSETT *et al.*, 1950). The following concentrations of these sugars were fed to groups of 100 flies each of which had been starved for 24 hours: 2·0 M D-glucose, 1·0 M L-fucose, 2·0 M D-mannose, and 1·0 M lactose. Figs. 1–4 show the effect of these feedings on the tarsal thresholds to glucose and fucose.

Feeding of any of the four sugars elevates both the glucose and fucose thresholds shortly afterwards. The duration of the period of elevation depends on the sugar fed. The rise in threshold is significant at the 1% level in all cases. Some of the differences between curves undoubtedly are a consequence of the different amounts of the sugars ingested, but the effect of mannose is of longer

duration than that of glucose although less mannose is ingested. A noteworthy point is the delay in threshold maximum after feeding, which is seen in the case of the mannose- and fucose-fed animals. Since fucose and lactose do not prolong life, threshold elevation upon ingestion of these sugars is not adaptive behaviour. The fucose-fed animals often die, presumably from starvation, before they respond to a concentration of glucose that would readily support life. Any explanation

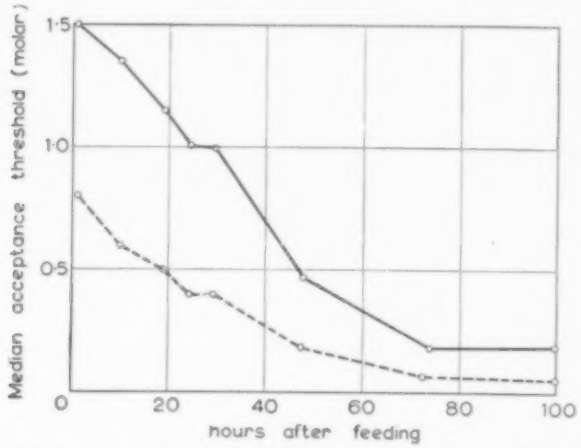

FIG. 1. Change in sugar thresholds following ingestion of glucose. Fed 2·0 M glucose; —— glucose threshold; - - - fucose threshold.

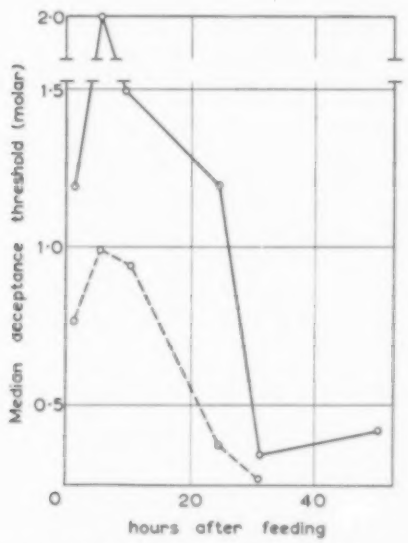

FIG. 2. Change in sugar thresholds following ingestion of fucose. Fed 1·0 M fucose; —— glucose threshold; - - - fucose threshold.

for the threshold elevation must account for the long duration of elevation, especially that of mannose.

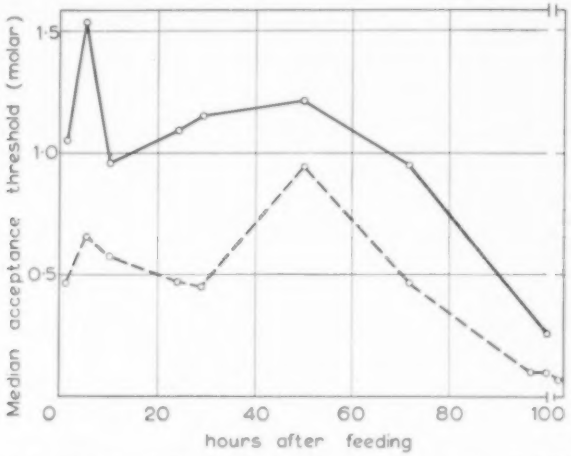

FIG. 3. Change in sugar thresholds following ingestion of mannose. Fed 2·0 M mannose; —— glucose threshold; - - - fucose threshold.

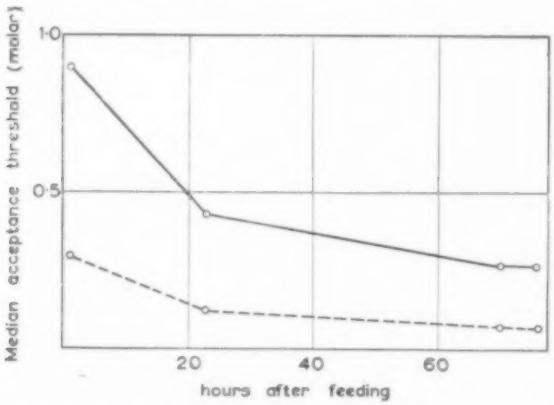

FIG. 4. Change in sugar thresholds following ingestion of lactose. Fed 1·0 M lactose; —— glucose threshold; - - - fucose threshold.

Three general ways in which elevation of threshold might be brought about by feeding of a sugar solution were considered: (1) action of the sugar on the external receptors only, producing a long-lasting refractoriness to stimulation by sugar; (2) the response of unknown receptors in some part of the digestive tract to sugars, to distension, or to some other stimulus which leads directly or indirectly to a central inhibition of the proboscis response; and (3) some internal action of the sugar itself or a metabolic derivative of it which eventually affects the nervous system.

The first hypothesis, a prolonged adaptation, seems unlikely from what little is known of the adaptation of the sugar chemoreceptors of insects. MINNICH (1929) indicated that disadaptation to the lower concentrations of sucrose was complete in less than 15 minutes. The experiments of VON FRISCH (1934) on the bee demonstrated clearly that disadaptation to concentrated sugar solutions was complete in less than 10 minutes. In addition, it is common experience when testing thresholds by random sampling or ascending technique that the same threshold will be obtained at the second testing if a 10- to 15-minute period intervenes. For these reasons it seems altogether unlikely that stimulation of the external chemoreceptors with sugar produces any effect peripherally or centrally that endures for more than 15 minutes.

Crop involvement

If any portion of the gut was involved, the most likely region was the crop. It was thought improbable that a sugar solution remained in the crop long enough to account for the prolonged high threshold observed after feeding

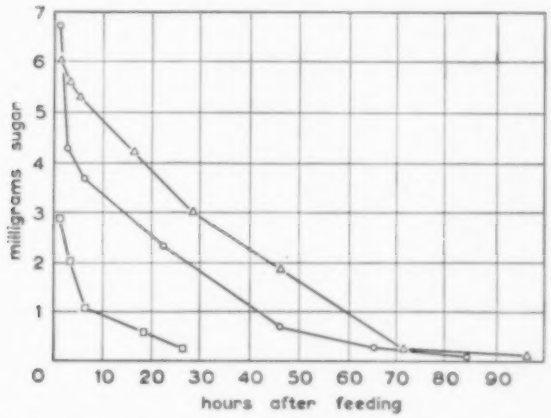

FIG. 5. Rate of removal of ingested sugars from the crop. Crop sugar after feeding. ○——○ 2·0 M glucose; □——□ 1·0 M fucose; △——△ 2·0 M mannose.

glucose and mannose. To check this, the amount of sugar remaining in the crop at successive times after feeding was measured (Fig. 5). Crop weight was also determined as a measure of volume. The last two sets of values merely parallel the crop-sugar values, indicating as might be expected that the crop concentration remains the same as the crop is emptied.

The rate of removal constantly diminishes with time and is slower in general for mannose than for glucose and fucose. Some fucose-fed animals die before the crop is depleted of sugar, indicating, in all probability, that their carbohydrate stores were low at the time of fucose feeding. In all three cases crop sugar becomes negligibly small at about the time sugar thresholds are minimal.

A more direct test of crop involvement was applied after some difficulty by ligation of the duct. It was found that the operation itself had no effect on threshold. This finding made it possible to ligate the crop and then test the effect of feeding of 2·0 M glucose on sugar threshold. Much less glucose was ingested under these conditions, but by colouring the solution to be ingested with methylene blue one could see that a considerable amount entered the mid- and foregut. None passed the ligature into the crop. The sugar threshold was markedly elevated by feeding. In one experiment 7 flies which had responded twice in succession to 0·125 M sucrose after ligation, for 3 hours would not respond to less than 1·0 M sucrose. Three of these flies would not respond even to 2·0 M. The crop, therefore, need not contain sugar solution for the act of feeding to elevate the sugar threshold for a prolonged period.

Almost identical results were obtained when the flies were fed 2·0 M glucose before crop ligation. In this case the other gut regions contained some of the fed sugar, just as they do when ligation precedes feeding. In both of these experiments the threshold began to fall again 4 to 6 hours after feeding, probably because the small volume of sugar in other regions of the gut had been depleted (see below).

Similar experiments have been reported briefly by BOLWIG (1952) using houseflies. Ligation of the crop and removal of an empty crop did not impair the "hunger reaction" (turning towards a drop of sugar solution when it touched the tarsus). Removal of a full crop and removal of the abdomen after a meal did not restore the hunger reaction for an hour or two. BOLWIG's conclusions agree with ours in that crop contents do not determine response to sugar solutions. The brevity of his report prevents interpretation of his interesting experiments of ligation anterior to the proventriculus.

It might be mentioned here, incidentally, that duration of feeding was measured in some of the animals whose crop duct had been ligated prior to feeding. Although they could ingest only a small fraction of the usual volume, these animals continued to feed for periods comparable to those of normal flies as previously reported (DETHIER *et al.*, 1956). This result is another indication that duration of feeding is regulated by adaptation of the sugar receptors.

Blood-sugar levels

We next investigated blood sugar in relation to sugar threshold. When blood sugar was first investigated, an unknown carbohydrate appeared on chromatograms, moving with known disaccharides in several solvent systems. After elution it was nonreducing but anthrone-positive. Surprisingly, the first determinations indicated a concentration of more than 10 g/l. in the blood. This component was always present in greater amounts than any other sugar and was present consistently, while the presence of other components depended upon which sugar was fed. When hydrolysed with acid it gave rise to glucose exclusively. Only three disaccharides of glucose linked between the first carbons are possible: α-α, α-β, and β-β. Only one of these is known to occur naturally, trehalose, 1-(α-D-glucopyranosido)-α-D-glucopyranoside. At this point in the investigation

a report appeared (WYATT and KALF, 1956) which described the isolation and chemical identification of trehalose from the blood of *Telea polyphemus*. Since the fed sugars appear in the blood as such in at least small amounts (glucose, fucose, mannose, lactose, and sucrose have been tested), the concentration of trehalose, the fed sugar, and any product of the fed sugar must be considered as having a possible effect on sugar threshold.

Since anthrone is said to be specific for substances convertible to a furfural derivative by sulphuric acid, total carbohydrates can be measured by the anthrone method by expelling blood into deproteinizing solution (5% trichloroacetic acid). The values thus obtained for adults range between approximately 2 g/l. and more than 30 g/l. (200 to 3000 Mg %). If a similar blood sample from glucose-fed animals is chromatographed and the chromatogram serially eluted in horizontal strips, anthrone-positive materials are found confined to three zones: the application zone, the trehalose zone, and the glucose zone. (A clean piece of Whatman 1 paper was developed simultaneously as a blank.) The sum of these values is the same or very nearly the same as the total anthrone value of deproteinized blood. Glucose may reach a maximum concentration of about 6 g/l., and trehalose ranges from about 1·25 to 30 g/l. The basal zone may represent glycogen; it does not vary significantly with feeding and has not been found at a concentration of more than 0·7 g/l.

Larval blood, in preliminary studies, seems very different. The total anthrone value is only 1 to 1·6 g/l. (as glucose) in the prepupal stage. Chromatographically there is seen to be no measurable trehalose; glucose accounts for 0·7 to 1·25 g/l. and the basal zone for about 0·35 g/l. A few studies on earlier larval stages indicate a similar picture; glucose is present to a variable extent, and trehalose is either very low or absent.

The adult blood contains very few cells; when a 20–50μl sample of blood is centrifuged in a microtube, no appreciable sediment occurs. When the supernatant from blood centrifuged at 3000 r.p.m. for 15 minutes is chromatographed, all three typical carbohydrate regions are present. Larval blood, however, contains many fold the cells of adult blood. These are largely granule-laden amoebocytes. The extremely rapid clotting of larval blood is probably a consequence of this high cell number. The contribution of the larval cells to their blood carbohydrates has not yet been evaluated.

Although the spray reagent employed was capable of producing colour with 2–5μg of glycerol, 20μl of adult blood did not exhibit any colour in the glycerol zone of the chromatogram.

Though WYATT and KALF (1956) reported qualitative detection of trehalose in eight species of insects and we have found it in adult *Phormia* and adult bees (see below), the few studies so far do not allow generalization to all insects. LEVENBOOK's (1950) analysis of *Gastrophilus* blood detected no blood carbohydrates with the properties of trehalose, although his study was quite thorough. Fructose was one of the main blood carbohydrates. Furthermore, there is the apparent lack (chromatographically) of trehalose in larval *Phormia* blood and in

the prepupa of *Isia isabella*. It is interesting to note in this regard that trehalose does not fulfil the definition of "true blood sugar" sought by previous investigators (fermentable reducing sugar) since it is not detected by any of the usual reducing methods. In fact even its fermentability is questionable although yeast stores rather large amounts of trehalose (O'CONNOR, 1940; BOUTHILET *et al.*, 1949; NARD and WEISS, 1951). The omnipresence of a low glucose value in adult and larval blood quite independent of glucose in the diet makes it necessary to consider glucose an important blood sugar also, especially in larval stages. The level of blood glucose is such that previous determinations of fermentable reducing sugar in a multitude of insects (cf. BEUTLER, 1939; and BUCK, 1953) may mean that glucose is present in low concentrations (relative to trehalose) in all insects.

In view of BEUTLER's (1939) report of up to 2000 mg % fermentable reducing sugar in honeybee blood, it was thought that this insect might lack blood trehalose. However, chromatograms indicated considerable quantities of trehalose as well as glucose and fructose. In the few cases studied no sucrose was observed. Those bees which were collecting pollen were noted to have little or no sugar other than trehalose in the blood, while those collecting nectar contained large quantities of glucose and fructose.

Changes of blood sugar with feeding

Figs. 6, 7, and 8 represent the effect of feeding 2·0 M glucose, 1·0 M fucose, and 2·0 M mannose on the blood carbohydrate composition. In the case of

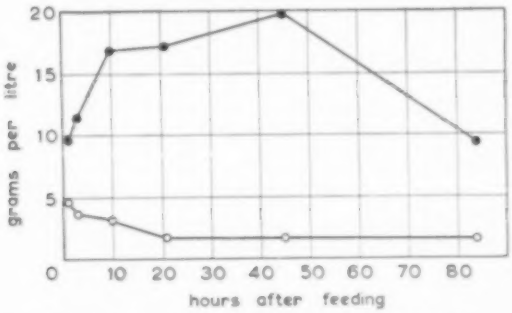

FIG. 6. Blood-sugar levels after glucose ingestion. Fed 2·0 M glucose; ●——● blood trehalose; ○——○ blood glucose.

mannose feeding (Fig. 8) the curve labelled mannose represents both glucose and mannose. These were read together since these sugars were difficult to separate sufficiently well for quantitative determination. The true blood mannose values are probably 0·5 to 0·7 g/l. less than shown on the graph. In all the experiments the various sugars are expressed as absolute units of that sugar and not relative to any other sugar (*e.g.* "as glucose") unless so stated.

The dietary sugar in the three cases reached rather high values in the blood. In the case of glucose and mannose the blood concentrations were regulated to

much lower values at the end of 20 to 30 hours, while blood fucose increased until death (30–40 hours). The fall of blood trehalose after fucose feeding

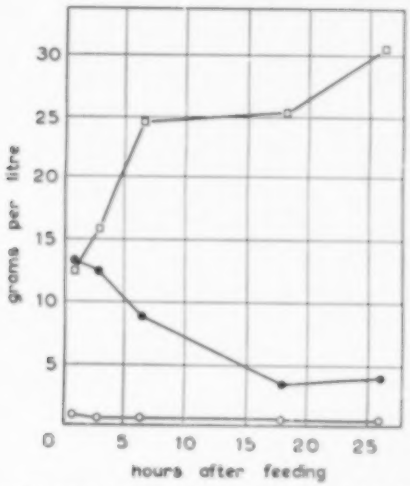

FIG. 7. Blood-sugar levels after fucose ingestion. Fed 1·0 M fucose; ●——● blood trehalose; ○——○ blood glucose; □——□ blood fucose.

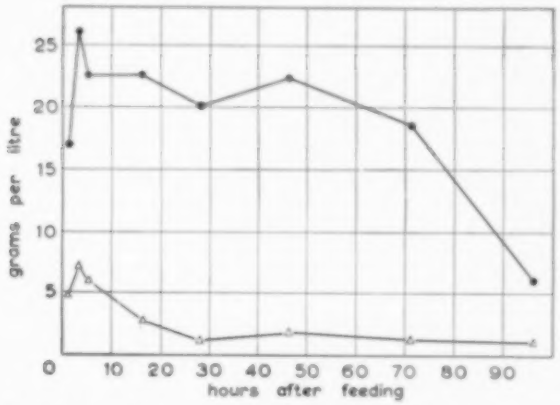

FIG. 8. Blood-sugar levels after mannose ingestion. Fed 2·0 M mannose; ●——● blood trehalose; △——△ blood mannose.

illustrates the strict dependence of that sugar on nutritional state. Blood trehalose after glucose and mannose feeding bore a simple relation to the curves for sugar threshold and for crop sugar. As the rate of crop emptying becomes very slow, blood trehalose begins to fall, and the sugar thresholds decline to normal values. Blood mannose and glucose do not appear likely threshold determinants since they assume nearly constant values while sugar threshold is radically decreasing.

Trehalose in those cases could be determining, but the threshold curve of the fucose-fed animals appears unrelated to any of the blood sugars.

Injection of sugars into the blood

As a more direct test of blood-sugar involvement, injections of glucose, trehalose, and fucose into the blood were performed. In several of the experiments determinations were made of the injected sugar in the blood. Each of the following sugar quantities was injected into 60 to 120 animals which had been starved 24 hours previously: fucose 0·858μl (250μg), 2·68μl (879μg); glucose 0·429μl (116μg), 0·858μl (60μg), 1·29μl (464μg), 2·68μl (968μg); and trehalose 1·34μl (509μg), 1·79μl (1220μg). When threshold to glucose was tested at several intervals after the injection (5 minutes to 24 hours), no significant (at the 1% level) rise in threshold occurred.

When 968μg of glucose was injected, blood glucose had declined at the end of 90 and 150 minutes to 1·9 l. and 0·30 g/l. respectively. Earlier times were investigated and the following values obtained: 10 minutes, 59·5 g/l.; 30 minutes, 42·6 g/l.; and 60 minutes, 25·9 g/l. When 509μg trehalose was injected, the blood-trehalose values were: before injection, 3·65 g/l.; 5 minutes post, 17·6; 20 minutes post, 17·0; and 60 minutes post, 12·1. At the same times the corresponding glucose thresholds were: 0·21, 0·30, 0·30, and 0·30 M. We were forced to conclude that these alterations of the level of several sugars in the blood, some to values similar to those achieved after feeding of sugars and others many times higher than such values, did not affect sugar thresholds.

The few blood-sugar determinations after injection, incidentally, constitute glucose and trehalose tolerance curves for *Phormia*. This fly clearly can withstand the introduction of phenomenal quantities of sugar into its blood when we consider that blood volume is probably only 4–6μl. The concentration of the injected sugar in the blood decreases rapidly, although not necessarily to original levels. In contrast blood trehalose maintains a maximal level for many hours after the feeding of a nutritive sugar. The level of blood glucose after feeding returns to normal in a much shorter period of time. Thus the change in concentration of blood sugars is not entirely passive in this fly, though the manner and time course of tl e regulation differ from those of mammals.

By elimination of the other possibilities, then, the locus of sugar action in elevating sugar threshold is some region of the gut other than the crop. We considered it impractical at the present time to attempt further localization. A number of hypotheses as to mechanism, which are based in part upon the continually decreasing rate of crop emptying, are possible.

Effect of flying on threshold

Continuous flying by *Phormia* following ingestion of 10μl of 1·0 M glucose was found to diminish radically blood trehalose and glucose (Table 1). The blood trehalose tends to recover during a subsequent rest period. Blood glucose in one experiment did not recover during the rest period, indicating that glucose

probably depends more upon the amount of ingested solution remaining in the gut than does blood trehalose.

We are grateful to Dr. Hudson of this laboratory for permission to quote certain results from a paper she is currently preparing. She found that sugar thresholds decreased during brief periods of flight (90 to 120 minutes) to levels

Table 1—Effect of flight on blood-sugar levels (g/l.)

		Before feeding	Before flight	After 90-min flight	After 2-hr recuperation
Exp. 1:	Blood glucose	—	2·43	0·33	0·73
	Blood trehalose	—	5·98	1·22	2·23
Exp. 2:	Blood glucose	0·33	3·01	1·49	1·14
	Blood trehalose	1·88	7·29	4·07	9·00

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that would not have been reached by starvation for several days. Furthermore, during subsequent recuperation periods of 1–2 hours thresholds remained low while, as seen above, the blood glucose and trehalose tended to increase. This finding supports the above evidence that blood sugar does not play a rôle in threshold determination.

Sugar thresholds of honeybees

Since the literature on the sugar thresholds of honeybees was unclear and did not fit our findings with *Phormia*, a few exploratory studies were made on bees collected in October 1956. The bees were mounted on wax sticks, and their tarsal thresholds were determined in the same manner as for *Phormia*. Care was taken so that mouthparts and antennae did not touch the solution. Altogether about 20 bees were studied; the results on the following 4 bees are typical. When these bees were collected, their thresholds for sucrose were 0·5, 0·125, 0·125, and 0·25 M. They were then fed 2·0 M sucrose. Their sucrose thresholds were then 2·0 M or higher for more than a day. One reached a threshold of 0·125 M at 45 hours and then died. The others reached low thresholds after longer periods (No. 1, 0·5 M at 67 hours; No. 3, 0·25 M at 74 hours; and No. 4, 0·0625 M at 67 hours). When again fed 2·0 M sucrose, they again became negative to all concentrations up to and including 2·0 M. Another bee, after being fed 2·0 M glucose, was flown for 1 hour on a thread waxed to the mesonotum. At the end of this period the threshold to sucrose had not declined. The following morning the threshold was still high, but a 20-minute flight brought the threshold down to 0·125 M sucrose. Hence the 80-minute flight sufficed to alter threshold from a value of more than 2·0 M to 0·125 M in 18 hours instead of the 45–74 hours required by starvation.

The crop and midgut of fed bees contained large quantities of the ingested sugar solution. Examination of the crops during starvation showed clearly that the bee will normally utilize ingested sugar solutions if its own energy reserves are depleted. If a foraging bee returns to the hive with a crop load of nectar for the hive, it is only because she collected in excess of the energy requirements for the return trip.

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AN EXAMINATION OF PROTEOLYTIC ENZYMES FROM SEVERAL INSECTS FOR COLLAGENASE ACTIVITY

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Abstract—Considerable collagenase activity was demonstrated in the excreta of aseptically grown blowfly larvae using a substrate (kangaroo-tail tendon) rich in undenatured, mature collagen. However, no evidence of such activity was observed in protease preparations from aseptically grown housefly larvae, from normally grown larvae of the webbing clothes moth and the silkworm, or from the American cockroach and the migratory locust.

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THERE are very few examples to support a commonly accepted view that, in insects, "adaptation to certain very specialized foods involves the presence of enzymes unfamiliar to mammalian forms" (CRAIG and HOSKINS, 1940). Those examples which are best founded deal mainly with the ability of some insects to degrade structural fibrous material, *e.g.* cellulose, keratin, silk, and collagen (DAY and WATERHOUSE, 1953), but, even in these instances, it has not always been possible to eliminate the doubt that bacteria may have played some part in the activity observed.

The early work of HOBSON (1931) established that the excreta of nonsterile blowfly larvae (*Lucilia sericata* Mg) contained an enzyme capable of digesting collagen (from bovine *tendo Achillis*). However, in recording a collagenase in sterile larvae, catgut was used as the substrate, and this would not now be regarded as constituting an entirely satisfactory source of unaltered collagen. Furthermore, MESSER and MCCLELLAN (1935) were subsequently unable to detect collagenase activity in sterile larvae. More recently collagenase activity was again reported in excreta of sterile *L. sericata* larvae, using coarsely ground collagen prepared from cattle hide (ZIFFREN, HEIST, MAY, and WOMACK, 1953). Since grinding tends to increase the digestibility of collagen (KENDREW, 1954), it appeared desirable to confirm the presence of a collagenase in the excreta of sterile blowfly larvae using a substrate rich in undenatured collagen. Furthermore, in order to investigate whether collagenase activity might be a property of many insect protease complexes, a comparison was made of the relative activities against collagen and other substrates of proteases from several insects which never normally ingest collagen.

It is interesting to note the recent report by LIENERT and THORSELL (1955) of a collagenase in second instar larvae of the warble fly, *Hypoderma bovis* (L.). It is probable from their occurrence in the bovine spinal column at this stage that the larvae were sterile and furthermore, from the methods employed in its preparation, that their collagen substrate was relatively pure.

Adequate evidence of ability to produce a collagenase is available for only a very small number of organisms, and there is good evidence that this is quite an unusual attribute even of bacteria. Thus, although the genus *Clostridium* includes some species or strains of species which produce an active collagenase, there are a larger number of *Clostridium* species unable to do so (MacLennan, Mandl, and Howes, 1953).

EXPERIMENTAL

A. *Sources of enzymes*

(i) *Larvae of Lucilia cuprina Wied., the Australian sheep blowfly.* A sterile protease preparation was obtained from larvae grown to maturity (3 days) at 30° C under aseptic conditions on an artificial medium (Waterhouse and Stay, 1955). The semifluid food residue (referred to herein as excreta) was diluted with a small amount of sterile distilled water and filtered through sterile organdie. After absence of contaminating organisms had been established from smears and from inoculations on nutrient agar, the filtrate was centrifuged to obtain a clear, dark-brown supernatant.

(ii) *Larvae of Musca domestica L., the housefly.* Sterile excreta were obtained as for *Lucilia* except that immersion of the separated eggs for 2½ hours in 5% Dettol* was necessary to ensure surface sterility.

(iii) *Large nymphs and adults of Periplaneta americana L., the American cockroach.* The alimentary canal was dissected from nonsterile individuals, blotted and rolled on filter paper to remove most of the adhering blood and fat body, and cut into 3–4-mm lengths before centrifuging.

(iv) *Adult Locusta migratoria L., the migratory locust.* Midgut caeca were dehydrated with acetone, and the acetone was removed with ether. The defatted caeca were then air dried and extracted with water adjusted to pH 7·4 with NaOH.

(v) *Large larvae of Tineola bisselliella (Humm.), the webbing clothes moth.* Whole larvae were desiccated over P_2O_5 and defatted by Soxhlet extraction with diethyl ether before extraction with water adjusted to pH 7·4.

(vi) *Large larvae of Bombyx mori (L.), the silkworm.* These were bled by cutting off the tips of the prolegs and then anaesthetized with CO_2. The alimentary canal was removed, cut into several pieces, and centrifuged to obtain a fluid containing digestive enzymes.

(vii) *Soluble trypsin.* Commercial trypsin (Pfanstiehl) which had been extracted with ice-cold water, centrifuged at 0° C, and the supernatant freeze-dried.

All of the insect preparations were clear solutions, but the trypsin solution was slightly cloudy. Thymol was added to all preparations to inhibit bacterial growth. When the solutions were not used immediately after preparation they were stored in a deep-freeze unit.

B. *Enzyme preparations used*

Trypsin was dissolved in water, the pH adjusted to 7·4 with NaOH and the volume made up to a trypsin concentration of 40 mg per millilitre.

The insect proteases were all dialysed against continuously stirred 0·0067 M Sorensen phosphate buffer (KH_2PO_4–Na_2HPO_4) of pH 7·4 for 56 hours at about 4° C. The buffer solution was changed several times during dialysis. The total nitrogen figures for these preparations, estimated by the micro-Kjeldahl method, are shown in Table 1.

In early experiments some preparations of *Lucilia* and *Musca* were dialysed against 0·01 M borate buffer of pH 7·4, but, since no advantage was apparently gained (the activities against collagen were slightly lower), the use of borate buffer was discontinued.

* Reckitt and Colman (Aust.) Ltd.

TABLE 1—TOTAL NITROGEN FIGURES FOR THE ENZYME PREPARATIONS USED

Enzyme preparation	mg N/ml
Trypsin	3·24
Lucilia	0·77
Musca	0·78
Periplaneta	2·42
Locusta	1·44
Tineola	4·24
Bombyx	0·58

C. *Substrates*

Kangaroo-tail tendon (KTT) was the air-dried tendon from kangaroo tail.

Kangaroo-tail tendon—citrate extracted. This had been prepared by extracting KTT with citrate-phosphate buffer of pH 4·0 (BRITTON, 1942) under toluene for 11 days at room temperature. The buffer was decanted, the citrate-extracted KTT washed and air dried. Both of these tendon preparations and the trypsin described earlier were the same as those used by SPRINGELL (1955). The tendon was cut into pieces about 3 mm long before use.

Azocoll was prepared from hide powder by the method of OAKLEY, WARRACK, and VAN HEYNINGEN (1946).

Azocasein was prepared from casein by the method of CHARNEY and TOMARELLI (1947).

D. *Activity estimations*

(i) *Activity against KTT and citrate-extracted KTT.* To 25 mg of substrate was added 1 ml of 0·067 M phosphate buffer pH 7·4 and 1 ml of enzyme solution. Incubation was carried out in beaker-covered test tubes at 37° C for 23 hours and then 1 ml 15% trichloroacetic acid was added. The tubes were left at about 4° C for 72 hours and any precipitated protein removed by centrifuging. Trichloroacetic acid-soluble nitrogen was estimated in the clear supernatant by the micro-Kjeldahl method.

Two controls were performed in parallel with test estimations (*a*) for soluble nitrogen in the collagen, in which the tube contained water in place of the enzyme solution, and (*b*) for soluble nitrogen in the enzyme solutions by omitting the substrate. The total amount of nitrogen in both controls was subtracted from nitrogen values for the test series in order to obtain the net value due to enzymic breakdown of the substrate. The amount of collagen digested was calculated assuming an 18·6% nitrogen figure (BOWES and KENTEN, 1948). All estimations were performed in duplicate and mean values recorded.

Digestion for 23 hours was chosen when insufficient breakdown was obtained after 2 and 4 hours to ensure that the results were not entirely due to digestion of non-collagenous material in the substrate. Thymol was added to all tubes to inhibit bacterial growth.

(ii) *Activity against azocoll.* Azocoll (25 mg) was suspended in 0·067 M phosphate buffer pH 7·4 (4·9 ml) and enzyme solution (0·1 ml) added. After shaking the wide mouth tube at 37° C for 15 minutes the azocoll was removed by filtration through Whatman No. 1 filter paper. The optical density of the filtrate was then read at 540 mμ on a Coleman Junior spectrophotometer. Relative activities were obtained from the degree of dilution of enzyme solutions required to give similar spectrophotometer readings (0·200 ± 0·010). The control reading (0·1 ml of water was substituted for the enzyme solution) was subtracted from test values.

(iii) *Activity against azocasein.* The method of CHARNEY and TOMARELLI (1947) as modified by DAY and POWNING (1949) was employed, using a buffer solution of pH 7·4. The optical

density at 440 mμ of the solution was determined with the spectrophotometer and the relative activities of the enzyme preparations estimated as for azocoll (reading 0·380 ± 0·020).

TABLE 2—COLLAGEN DIGESTION BY INSECT ENZYME PREPARATIONS

Enzyme preparation	KTT digested		Citrate-extracted KTT digested	
	mg	%	mg	%
Trypsin	1·9	7·6	1·3	5·2
Lucilia	8·9	35·6	8·4	33·6
Musca	0·6	2·4		
Locusta	1·4	5·6		
Periplaneta	2·4	9·6		
Tineola	0·4	1·6		
Bombyx	0·2	0·8		

RESULTS AND DISCUSSION

The extent to which the KTT substrates were attacked by the various enzyme preparations is shown in Table 2. The control for KTT (without enzyme) averaged 0·04 mg and for citrate-extracted KTT 0·08 mg nitrogen. The controls for enzyme preparations varied from 0·35 mg for *Bombyx* to 3·12 mg nitrogen for trypsin. However, in spite of this high figure for trypsin, 10% of its initial activity against azocasein was still present after 23 hours' incubation. This activity is equivalent to half that shown initially by the *Lucilia* preparation (Table 3).

If it is assumed that the collagenase activity of trypsin is negligible under our conditions (KENDREW, 1954), it would appear that proteins other than mature collagen comprise about 7·6% of KTT and about 5·2% of citrate-extracted KTT. Whether or not this assumption is made, it is clear that the *Lucilia* preparation contains a very active collagenase, thus confirming the reports of HOBSON (1931) and ZIFFREN *et al.* (1953) of the presence of this enzyme in blowfly larvae.

TABLE 3—RELATIVE ACTIVITY OF INSECT ENZYME PREPARATIONS AGAINST DIFFERENT SUBSTRATES

Enzyme preparation	Relative activity*		
	Azocasein	Azocoll	KTT
Trypsin	100	100	100
Lucilia	20	60	470
Musca	1	3	30
Locusta	25	90	80
Periplaneta	25	110	130
Tineola	4	10	20
Bombyx	5	15	10

* Relative activity expressed as percentage of trypsin activity. It should be noted that the percentage digestion of each substrate by trypsin was different, so that figures can only be compared directly within vertical columns.

When figures (Table 3) for the relative activities of the various preparations against azocasein, azocoll, and KTT are considered, this conclusion is supported. With the possible exception of *Periplaneta*, no evidence of collagenase activity was obtained for the five other insect proteases. When the *Musca* enzyme preparation was concentrated to about one-third of its volume (by desiccation over P_2O_5 in the refrigerator) its activity against KTT relative to trypsin rose to about 40. It is clear that if a collagenase is possessed by these five insects, it could only be demonstrated by using far more active preparations than were at our disposal. In addition, preparations would have to be obtained in each instance from insects reared under aseptic conditions. It is interesting to note that housefly larvae (like blowfly larvae) discharge proteolytic enzymes with their excreta although we did not obtain a highly active preparation from this source.

Acknowledgement—Dr. P. H. SPRINGELL, Biochemistry Unit, Wool Textile Research Laboratory, CSIRO, Melbourne, kindly supplied the trypsin, kangaroo-tail tendon, and citrate-extracted kangaroo-tail tendon used in these experiments.

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STUDIES ON ARTHROPOD CUTICLE—XIII

THE PENETRATION OF DISSOLVED OXYGEN AND ELECTROLYTES IN RELATION TO THE MULTIPLE BARRIERS OF THE EPICUTICLE*†

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Abstract—Using isolated cuticles of *Sarcophaga* larvae, the penetration of oxygen was measured polarographically and the penetration of ions was studied by complex impedance. Comparison of data from normal and treated cuticles shows that the heterogeneous epicuticle contains at least three distinct components. The ionic barrier is the exceedingly thin outer epicuticle which is resistant to a variety of hot solvents, detergents, and acids but is disrupted by alkali. The water barrier is located in the thicker inner epicuticle (appears to be the outer portion thereof), and is disrupted by organic solvents. The site for control of asymmetrical penetration is in the inner portion of the inner epicuticle, and is not disrupted by hot solvents. A small amount of data on other species suggests that in general the electrolyte barrier will be found to be distinct from the water barrier in insect cuticles. It follows that one of the reasons for the superposition of numerous sublayers in the insect cuticle is that different sublayers are barriers to different groups of chemicals. Brief note is made of the fact that the electrical properties of cuticle were found to be complicated, and to include rectification, polarization effects, and even bimodal polarization peaks which imply transient energy changes.

INTRODUCTION

THE classical experiments of WIGGLESWORTH (1945) and BEAMENT (1945) showed that in general among insects a wax sublayer in the epicuticle constitutes the effective barrier to passage of water (transpiration). But larvae of higher Diptera possess no wax layer (DENNELL, 1946 ; WOLFE, 1954) yet are resistant to desiccation and to the penetration of various solutes (RICHARDS and FAN, 1949). Data have already been published showing that the penetration of water (WOLFE, 1955) and the property of asymmetrical penetration (RICHARDS *et al.*, 1953) are both controlled by the inner epicuticle (= cuticulin layer) of fly larvae. The purpose of the present study was to locate the effective barrier to water-soluble solutes with particular reference to the cuticles of fly larvae.

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The author's sincere thanks are due to Dr. OTTO H. SCHMITT who assembled and made available the equipment for impedance measurements, and who advised throughout the work. Thanks are also due to Mr. KELSO MARSHALL and Mr. WM. BATTIN for much of the preliminary work in polarography.

It was found that the effective barrier to water-soluble compounds in fly larvae is not the same as the barrier to water itself, and, further, that the same appears to be true also for insects which possess a wax sublayer in the epicuticle.

MATERIALS AND METHODS

Sarcophaga bullata, used for most of the experiments, is maintained as a colony in the laboratory, the larvae being reared on ground beef. At our insectary temperatures larvae reach the pre-pupal stage about 7 days after larviposition. All larvae used were in the last larval instar, usually 4–5 days after larviposition. When used, larvae of the cranefly *Tipula abdominalis* and the neuropteron *Corydalus cornutus* were collected from local streams. Larvae of the wax moth *Galleria mellonella* and adults of the giant cockroach *Blaberus craniifer* were taken from stock cultures maintained in routine manner.

For preparation of isolated cuticles of *Sarcophaga*, larvae of known age were cut longitudinally, eviscerated, and gently swabbed on the inner surface with a soft brush while immersed in distilled water. They were allowed to soak in distilled water for 30 minutes to several hours with repeated swabbing to remove most of the muscles and epidermis (traces of tissue, especially attached ends of muscles, usually remain). In a few cases cuticles were scraped clean with a scalpel, rinsed and tested within a few minutes after killing the larva. Tests showed that there was no significant difference between the cuticles that were used immediately or those that were stored in the cold for several days or even several weeks. This isolation procedure appears to be valid because intact larvae show similar rates for oxygen penetration, and whole integuments give impedance curves similar to ones that can be obtained from the cuticle isolated from the same piece of integument. Fly larvae are especially favourable because they supply relatively large sheets of cuticle (1 cm² or more) free from gross discontinuities.

Oxygen penetration was measured by a polarographic method utilizing reduction onto a platinum cathode. Essentially the method was that of DAVIES and BRINK (1942) employing a flush-type electrode and a streaming system which eventually attains a steady state. The cell employed (Fig. 1) has a 0·5 mm platinum wire cathode (Pt) and a calomel anode (Cal) connected to a circuit to supply a

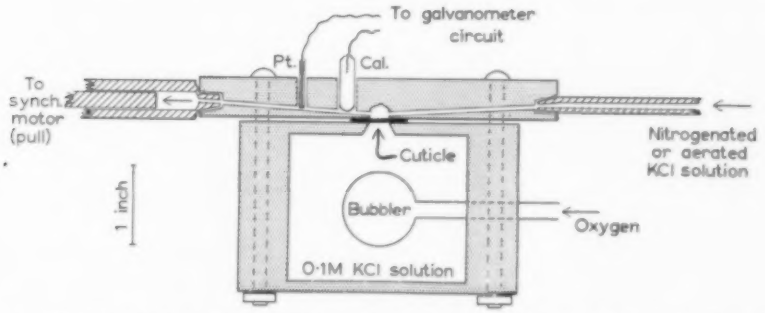

FIG. 1. Diagram of the cell and membrane used to measure the rate of oxygen penetration.

constant voltage of 0·65 V and to measure current flow. The d'Arsonval galvanometer used had a measured sensitivity of $2{\cdot}35 \times 10^{-11}$ amperes/mm. The cell was routinely filled with 0·1 M KCl solution of controlled gas content, and the rate of flow of the solution past the membrane and electrodes was measured during each experiment (usually about 0·1 ml/hr).

With this particular cell system and rate of flow approximately 2 hours are required to obtain a steady state that can be compared with values for air-saturated and nitrogen-saturated KCl solutions (Fig. 4). The system is sensitive to slight pressure changes, and mechanical difficulties with the plunger syringe used to control the flow usually resulted in our obtaining a somewhat sawtoothed distribution of points. This introduces some uncertainty about the absolute values but does not seriously affect the point that scratching produces considerable change whereas chloroform does not.

For electrolyte penetration two methods were used. Complex impedance circle diagrams (Figs. 5–6) were calculated from resistance and capacity measurements at various frequencies by means of a precision Wheatstone bridge circuit modified by use of a differential amplifier so that both cell and oscillator could be grounded. The circuit was nulled and the harmonic content monitored with an oscilloscope. Electronic high and low pass filters were arranged as a narrow band pass at the frequency used, and voltage across the cell was monitored and held at 0·15 RMS volts. The lowest output of the oscillator employed was 16·7 cycles/second. Usually a series of measurements were made from this value to several kilocycles, but for cuticles treated in NaOH solutions measurements were made at values as high as 200 kilocycles. The KCl solution usually used for filling the cell was made to 0·19 M because this approximates the osmotic pressure of fly blood.

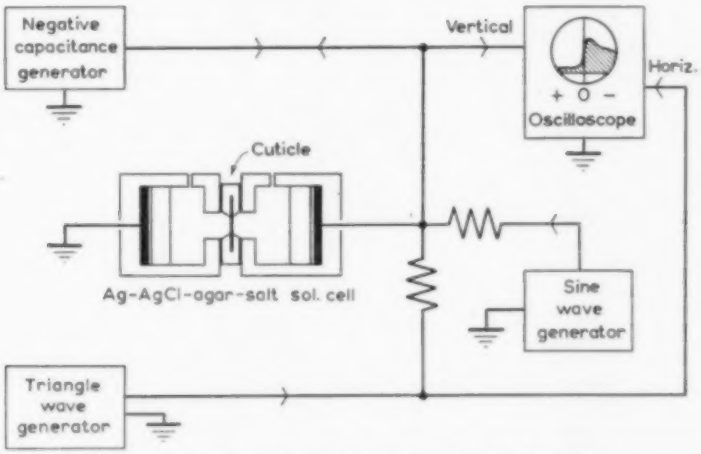

FIG. 2. Block diagram of the equipment used for dynamic impedance measurements. For a more detailed diagram see SCHMITT (1955). The cell is constructed of Lucite; the membrane holder of Teflon.

As an outgrowth of the above work, Dr. O. H. SCHMITT developed a new method to allow extension of the analysis. A simplified block diagram of the apparatus and cell is given in Fig. 2. The oscilloscope shown plots a.c. resistance to a small test signal as a function of d.c. polarization which is continuously swept from $+30\mu$A through zero to -30μA. Electronically-generated negative capacitance is adjusted to cancel the static structural capacitance of the membrane, thereby revealing dynamic changes in membrane conductance produced by mild polarization and normally swamped by membrane susceptance. Other oscilloscopes not shown in Fig. 2 monitor the phase ellipse and static current-voltage characteristic. The method has already been described with functional block diagrams as well as representative photographs of the oscilloscope figures obtained (SCHMITT, 1955).

Appropriate tests show that electrolyte penetration does occur and that it is independent of the type and amount of gas dissolved in the salt solution.

With all of the above methods, but especially with the impedance measurements, the design of the cell and membrane holder was found to be critically important. Sealing against leakage between the two sides of the cell is essential. For the polarographic method adequate sealing was obtained with vacuum grease and some pressure from the clamping screws. For the impedance study we wished to avoid both grease and drying, and also to have a holder in which the membrane could be treated without the hazards of removing it from the holder. A special "Teflon" membrane holder was designed (Fig. 3), and in addition it was found necessary to

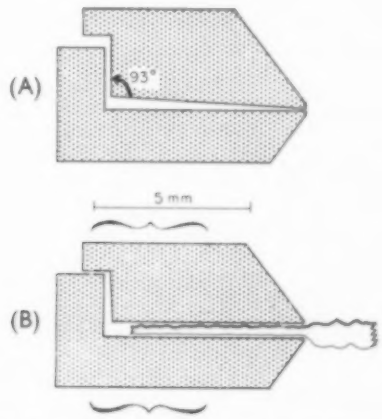

FIG. 3. Scale drawing of a section through one side of the circular Teflon membrane holder. (A) Empty holder at rest showing the 3° angle incorporated. (B) Holder with piece of cuticle in place as it appears when in the assembled cell with pressure being applied over the area indicated by brackets. Note compression of portion of cuticle between the half-holders.

assemble the half-cells in a frame of kinematic design to equalize pressure around the circular aperture. Note that the halves of the holder at rest are out of parallel by 3°. When pressure is applied in the area indicated by brackets, the surfaces

become parallel with extra pressure at the margin of the aperture. This is both to deter the membrane from being squeezed out and to give added assurance that the aperture really represents the area of cuticle exposed for penetration. Even with this holder the rugose epicuticular surface requires that several mm of cuticle be between the "Teflon" half-holders to assure nonleakage.

The holder used most frequently had an aperture of $\frac{1}{8}$ cm²; leakage difficulties were found to be more serious with holders having $\frac{1}{4}$ and $\frac{1}{2}$ cm² apertures, presumably because of a less adequate extent of membrane within the sealing area.

The adequacy of the pressure-sealing method has been questioned. However, the pressure can be varied experimentally; and the pressure routinely used was about 2× that necessary to give a constant value, presumably indicating complete sealing. Incidentally, if some leakage around the membrane did occur it would affect only the *absolute* electrical values, not the biologically interesting *relative* values used for locating and determining the properties of the ion barrier.

RESULTS

Penetration of oxygen

Analysis of plateau values after attainment of a steady state (Fig. 4a–b) shows that the normal isolated cuticle of *Sarcophaga* has an average penetration value of 0·06 c.c. O_2/mm²/hr/mole litre conc. difference (range 0·03–0·15 c.c. for 30 sets of measurements). This value is similar to values reported on the basis of oxygen consumption of ligatured larvae of *Calliphora* (FRAENKEL and HERFORD, 1938) and *Phormia* (BUCK and KEISTER, 1953). Perhaps this is a general value for last instar larvae of higher Diptera.

In two cases the cutaneous respiration of ligatured larvae was measured in Warburg vessels, and then the cuticles were isolated from these larvae and the oxygen penetration measured in the polarographic cell. In each the same rate was determined by the two methods (0·03 and 0·05 c.c. O_2/mm²/hr/mole). It follows that the cuticle alone constitutes the effective barrier. It also follows, as BUCK and KEISTER (1953) suggested, that the oxygen tension inside ligatured larvae rapidly falls to near zero.

With isolated cuticles, 19 sets of measurements were made with the oxygen penetrating in the direction epicuticle→endocuticle, 11 sets with penetration in the direction endocuticle→epicuticle. In about half of these sets the same cuticle was used for measuring the rate in both directions. No significant difference was found in penetration rates when the cuticle was wet with water on both sides. But the asymmetrical penetration reported for water by several investigators is also not obtained when both sides of the cuticle are wet with water (RICHARDS *et al.*, 1953).

Four isolated cuticles were treated with boiling chloroform for an hour and then the penetration rate measured. Three of these had previously been measured as normal cuticles. The hot chloroform produced no significant difference in the final steady state value (Fig. 4c).

Three isolated cuticles were measured, then the outer surface rubbed gently with fine sand or with emery cloth, and then the penetration rate remeasured.

Scratching the outer surface does increase the penetration rate of oxygen (Fig. 4d). The amount of increase is, of course, not constant since it is impossible to control the number of effective scratches produced.

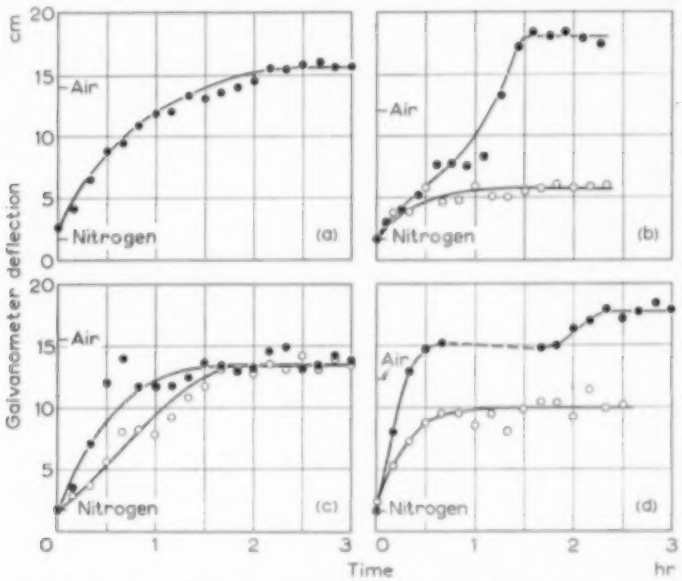

FIG. 4. Oxygen penetration rates through larval *Sarcophaga* cuticles as determined by reduction on a platinum cathode. Simultaneous values for air-saturated and nitrogen-saturated water are marked "air" and "nitrogen" on the vertical axis. (a) An example of gradual attainment of a steady state value for O_2 with diffusion through the cuticle from O_2-saturated to N_2-saturated solution. (b) An example of more definite plateaux for the steady state. Open circles represent diffusion from air-saturated to N_2-saturated solution; solid circles represent diffusion from O_2-saturated to N_2-saturated solution for the same cuticle. (c) Similar curves from one cuticle showing no effect from treatment with hot chloroform (open circles show original values; solid circles show values after treatment). (d) Similar curves showing the considerable increase in rate of O_2 penetration due to abrading the outer surface of the epicuticle (open circles show original values; solid circles show values after scratching, the interval shown by dashes being a period during which no readings were taken).

Despite the small number of treated cuticles used for measurement of oxygen penetration, it seems safe to conclude that the rate is affected by relatively slight mechanical injury to the integrity of the epicuticle but is not affected by extraction with hot chloroform.

Penetration of electrolytes

1. *The electrical properties of cuticles.* Using a Wheatstone bridge circuit one determines equivalent series or parallel resistance and capacity values at a chosen series of frequencies. Plotting series reactance values $(1/(2\pi fc))$ against series

resistance values on rectangular coordinates locates the terminals of the impedance vectors. When determinations are plotted for a series of frequencies, the points are found to fall along a semicircle (Fig. 5). A perfect semicircle is to be expected of

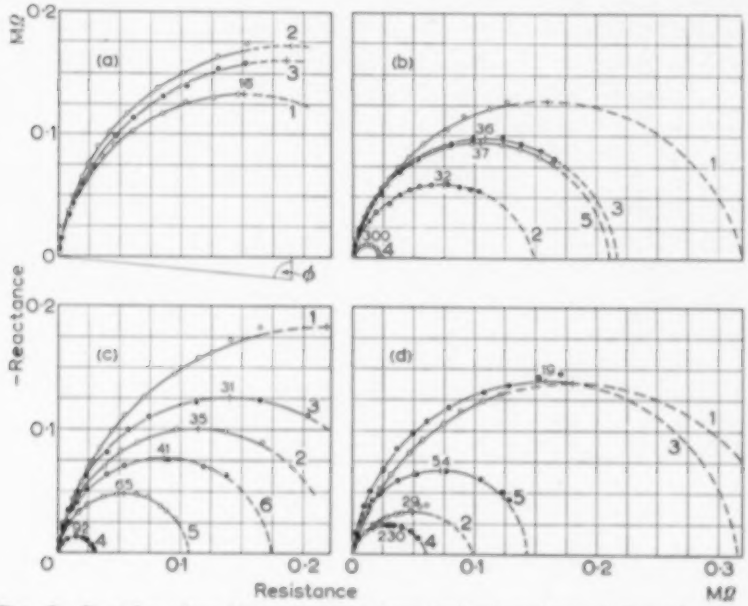

FIG. 5. Complex plane locus diagrams from isolated cuticles of *Sarcophaga* larvae. Curves beyond lowest frequency determination drawn as broken lines. Vertical bars at the top of each arc indicate the reactance maximum with the small number "characteristic frequency" when this is within the set of determinations. Curves are numbered in the sequence in which the different sets of values were determined and each set is from a single cuticle. (a) Curve 1 is the original set of values in 0·19 M KCl after the cuticle had soaked in cold distilled water for 2 days; curve 2 is the set of values 2 hours later; curve 3 is the set of values after refluxing first in chloroform (18 min) then in a chloroform-methyl alcohol mixture (20 min) with transferal to and from water via acetone. Phase angles 83–85°, that for curve 2 drawn in (ϕ). (b) Curve 1 is the original set in 0·19 M KCl after the cuticle had soaked in cold distilled water for 9 days; curve 2 is after refluxing in distilled water (30 min); curve 3 is after refluxing in petroleum ether (30 min); curve 4 is after soaking for 27 hr in cold 4 M KCl (measured in 0·19 M KCl); and curve 5 is after washing in boiling water (20 min) followed by refluxing with petroleum ether. Phase angles 78–89°. (c) Curve 1 is the original set in 0·19 M KCl after the cuticle had soaked in cold distilled water for 10 days; curve 2 is after refluxing in acetone (30 min) followed by refluxing in petroleum ether (30 min); curve 3 is after refluxing in methyl alcohol (15 min) followed by refluxing in petroleum ether (2 min); curve 4 is after refluxing in ethyl ether (10 min) followed by refluxing in dioxane (10 min); curve 5 is after refluxing in carbon tetrachloride (3 min) followed by refluxing in water (10 min) followed by boiling until dry in petroleum ether and then refluxing again in water; curve 6 is after refluxing in 1% aqueous "Aerosol OT" (40 min) followed by re.³uxing in petroleum ether (10 min). Phase angles 80–84°. (d) Curve 1 is the original set in 0·19 M KCl after the cuticle had soaked in cold distilled water for 18 hr; curve 2 is after refluxing in benzene (10 min) followed by soaking in 4% aqueous phenol at room temperature (5 days); curve 3 is after refluxing in benzene (10 min); curve 4 is after soaking in dioxane for 34 hr followed by boiling for 10 min followed by boiling in distilled water (2 hr); curve 5 is after another refluxing in benzene (30 min). Phase angles 75–87°.

an electrical circuit consisting of two resistors in series, one shunted by an ideal capacitor. Such a circuit is said to have a phase angle of 90° since the centre of the circle lies on the resistance axis. If, as with most biological membranes, the centre of the circle lies below the resistance axis (see Fig. 5a), then the phase angle (ϕ) is less than 90°. The more closely the phase angle approaches 90° the more satisfactorily the membrane can be represented by the simple model of two resistors and a capacitor. For further discussion of the method see COLE and CURTIS (1936).

When the above idealization is used to represent the membrane, isolated larval cuticles of *Sarcophaga* in 0·19 M KCl solution give a mean parallel capacitance and standard deviation of the mean of $0{\cdot}300 \pm 0{\cdot}0124$ μF/cm² (range for about 100 measurements = 0·141 — 0·540). Conductance of these same membranes was low and more variable than the capacitance, the extrapolated zero frequency resistance on the first measurement following isolation averaging about 40,000 ohms/cm² (range 15–60 kilo-ohms giving a mean of 38 ± 3).* Plotting values for various frequencies yields complex plane locus diagrams where the centre of the semicircle is consistently slightly below the axis (Fig. 5). The phase angle (ϕ in Fig. 5a) has a mean value and standard deviation of the mean of $82{\cdot}6° \pm 1{\cdot}2°$ (range 75–89°, and almost this whole range can be given at different times by a single cuticle, *e.g.*, Fig. 5d, 6). The "characteristic frequency" (the frequency at which the reactance value is maximal) is low for an untreated membrane usually slightly below the minimal output of our oscillator (16·7 cycles/sec).

From the above measurements one can deduce the already well-known fact that these cuticles have very low penetration rates for ions. The phase angle being large implies that the cuticle is more satisfactorily represented by the electrical model than is the membrane of various cell types. The capacitance of cuticle is also somewhat low, only $\frac{1}{4}$ to $\frac{1}{3}$ that of most plasma membranes, but this could be accounted for by any of several possible differences. It is of greater biological interest that one can also deduce that the effective barrier layer for ions is quite thin. When the condenser equation is applied, the calculated thickness falls in the range of 75–300 Å, or, within a factor of 2, averages 150 Å (see discussion section).

To our surprise, it was found that not only were there large changes in impedance values brought about by various treatments but that these changes were largely reversible. Thus, for the set of curves shown in Fig. 5b, it was found that the original zero frequency resistance value of 41,000 ohms/cm² was gradually reduced to 3000 by soaking in strong KCl solution and then returned to 27,000 by washing out the salt and temporarily dehydrating the cuticle with petroleum ether.† This represents changes from the original to 7·5% and then back to 66%

* Actually, considerably higher impedance values are usually (but not always) given by cuticles when measurements are first begun but the values fall for a time too rapidly to permit a valid set of determinations being made. The values stated here are for membranes after they had stabilized in the system, the time for stabilization varying from a few minutes to about an hour. Curves were considered valid when a repeat on the first determination at the end of a whole series gave approximately the same readings.

† These cuticles were never air-dried. They were always passed from aqueous to nonaqueous solvents *via* mutually miscible liquids, usually *via* acetone.

of it. In another example shown in Fig. 5d the original zero frequency resistance value of 44,000 ohms/cm² was lowered by treatment with aqueous phenol to 12,500, raised to 37,500 by hot benzene, lowered to 7500 by hot dioxane, and then raised back to 18,500 by a second treatment with hot benzene. This represents successive changes from the original value to 28%, to 85%, to 17%, to 42%. With decreasing resistance, the "characteristic frequency" rises, as is to be expected, to a maximum of several hundred cycles per second. Obviously the ionic barrier component of the cuticle is far from static. It is a layer capable of considerable hydration-dehydration and sorption-desorption of salt with corresponding effects on membrane conductance (and hence on ionic penetration). Discrepancies between expected and determined effects of changes in electrolyte concentration are presumably related to this phenomenon. Qualitatively similar hydration-dehydration effects were obtained with isolated larval cuticles of *Corydalus* and *Galleria*.

The data in the preceding paragraph provide a ready explanation for another phenomenon which would otherwise seem anomalous. It was found experimentally that the conductance of an isolated cuticle is much lower than the conductance of the living integument of which the cuticle was a part. In other words, the intact integument is more permeable to ions than is a piece of cuticle taken from it. This is graphically shown in Fig. 6, where the initial impedance values of the living integument (curve 1) rise as the cells presumably die (curve 2) and rise much further when the cells are killed by distilled water before measurement in KCl

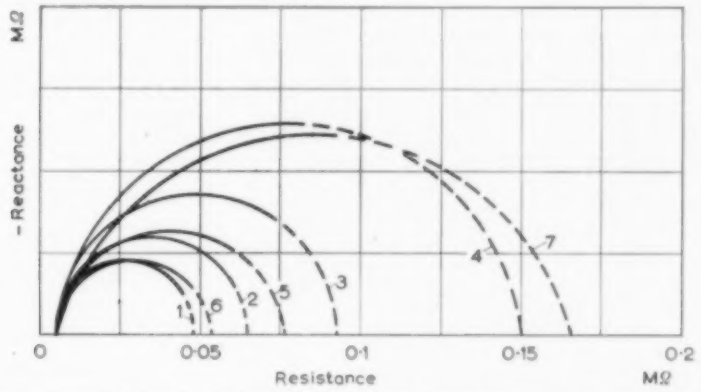

FIG. 6. Complex plane locus diagrams of the living integument of a *Sarcophaga* and of the cuticle isolated from it. For clarity of the overlapping curves, the individual points have been omitted but they fit the arcs as well as those in Fig. 5. Portions of the arcs beyond actually determined points are indicated by a broken line. Membrane area ¼ cm². Curve 1 is the original set in balanced saline solution; the attached heart was still beating after the determinations had been completed; curve 2 is the same about an hour later when the cells were presumably dying (heart inactive); curve 3 is the same in 0·19 M KCl solution after soaking 10 minutes in distilled water; curve 4 is the same about 2 hours later; curves 5 and 6 are the same after soaking in 0·19 M KCl for respectively 8 and 34 hours; and curve 7 is after boiling in distilled water for a few minutes followed by refluxing in chloroform (15 min). Phase angle of the living integument nearly 90°, for curve 3 it is 88°, then it falls to about 83° for curves 4–6, and to 75° for curve 7.

solution (curves 3–4). The elevated impedance can then be progressively lowered to approximately the original integumental value by prolonged soaking in KCl solutions (curves 5–6), and then returned to a high value by treatment to remove the salt and water (curve 7). (Incidentally, a similar increase of about 2× in impedance value was shown by the cuticle of the cockroach *Blaberus* on isolation from the living cells.) Clearly, the electrolyte barrier is not only labile but is somehow held at an intermediate impedance value and hence intermediate permeability value while the cuticle is in place on either a fly larva or a cockroach adult.

Even with the simple Wheatstone bridge circuit, additional electrical properties are evident. Thus electrical nonlinearity of the membranes necessitated monitoring and adjusting the voltage across the cell to a chosen constant value, and harmonics generated by the membranes made the incorporation of high-pass and low-pass filters desirable. But these additional properties are best studied with another type of instrument such as that shown in Fig. 2. Analysis with this new method is incomplete. Accordingly, only a list will be given here to indicate the much greater complexity that will be encountered in a full analysis of the electrical properties of insect cuticle. Isolated *Sarcophaga* cuticles may be indifferent to a superimposed polarization or they may show sharp conductance minima either at zero polarization (Fig. 7a–b) or at a finite polarization value (Fig. 7f–h), depending on the history and ionic environment of the cuticle. Commonly, the polarization produces a different pattern when the sweep of polarizing current passes in opposite

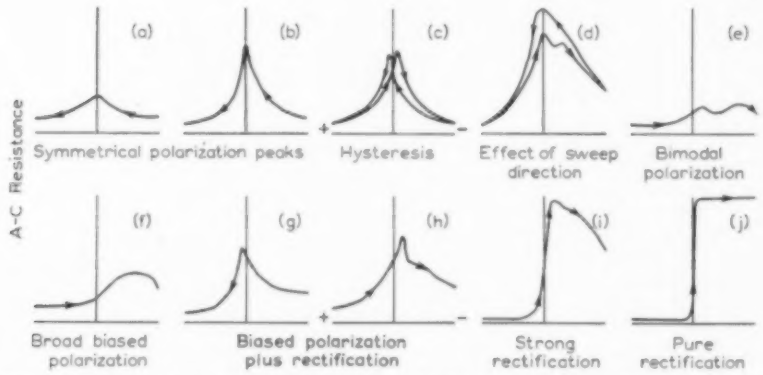

FIG. 7. Tracings from oscilloscope patterns obtained from isolated *Sarcophaga* cuticles by the dynamic impedance method. The vertical axis plots a.c. resistance to a small 100-cycle test signal with the total height of the axis equal to 37,500 ohms/cm². The horizontal axis plots d.c. polarization current which is swept continuously by a triangle generator from +30 microamperes through zero to −30μA and back again. The + and − signs on the tracings refer to the epicuticle side of the cuticle. Arrows superimposed on the curves indicate the direction of sweep for that curve. Curves a–i were with solutions whose electrolyte concentration had been adjusted so that each solution had the same conductance.

Curves a and g with $CaCl_2$, curves b, e, and h with KCl, curves c and d with NaCl, curve f with KF, curve i with Na barbital, curve j with dilute NaOH on the epicuticle side and dilute HCl on the endocuticle side (the difference in resistance shown is about 25×).

directions through the cuticle (Fig. 7d).* In some cases with certain salts (especially if the membrane is fresh) the cuticles even show bimodal conductance peaks implying transient energy changes (Fig. 7d–e). The isolated cuticles also show strong rectification (like living squid nerves), the amount of which depends on the nature of the ionic environment. Rectification is maximum and pure with a dilute HCl solution on one side of the cuticle and a dilute NaOH solution on the other (Fig. 7j), but fairly good rectification effects can be got with some single salts present in the same concentration on both sides of the cuticle (Fig. 7i). There are, then, not only asymmetrical effects, but several kinds of asymmetrical effects. And the differences appear to be related to the state and history of the cuticle more than to either the species or concentration of ions in the medium. Incidentally, although these changes under changing polarization are fairly rapid, there is enough time-lag so that considerable hysteresis is evident (Fig. 7c).

2. *Chemical properties of the electrolyte barrier.* The thin barrier layer is extremely resistant chemically. As pointed out in the preceding section, it may be reversibly modified in conductance by various treatments that do not destroy it (Fig. 5). Listing the treatments given: the barrier is not disrupted by boiling for some minutes (usually 5–15 min but sometimes longer) in distilled water, chloroform, benzene, toluene, xylene, petroleum ether, carbon tetrachloride, ethyl ether, dioxane, methyl alcohol, a 1 : 1 mixture of methyl alcohol+chloroform, acetone, or 0·01–1·0% aqueous solutions of the nonalkaline detergent "Aerosol OT" (Bis, 2-ethylhexyl, sodium sulfosuccinate). In some cases the impedance value is lowered (Fig. 5b–c), but in other cases it is not significantly affected (Fig. 5a), or it may be returned to approximately the original high value (Fig. 5d, 6). The barrier is also not disrupted by 5 days' soaking in 4% aqueous phenol, and it is only modified, not irreversibly changed, by 1 N nitric acid even on heating to boiling for about a minute. It was not changed by soaking for 32 hours in 0·1 N hydrochloric acid at room temperature. Obviously, it is not disrupted by heating to slightly over 100° C. It is also not disrupted by freezing.

However, the electrolyte barrier is completely and irreversibly disrupted by a few minutes' boiling in 10% or even 1% sodium hydroxide. The membranes giving the curves shown in Fig. 5a, c, and d were given their final treatment in hot alkali. The resulting cuticle still gave a circle diagram (thereby showing slight resistance to ionic penetration), but the semicircle is too small to draw on this scale, the impedance value having been reduced to a small fraction of 1% of the original. The barrier is readily disrupted by alkali applied either to the inner or to the outer surface of the cuticle; in other words alkali has effective access from either side of the barrier. The barrier is also quickly disrupted by heating in a solution of an alkaline detergent ("Alconox"). The barrier is more slowly disrupted by weaker alkaline solutions at room temperature (40 hr in 0·1% NaOH almost abolishes it).

* This unexpected influence of a polarizing current can be shown with complex locus diagrams, but it would be both laborious and limited to attempt study by this method. The fact is mentioned here only to say that the phenomenon can be demonstrated with this classical method as well as with the new dynamic impedance technique.

In a single test made with a strong oxidizing agent, the barrier was destroyed in 30 hr at room temperature in 1% aqueous potassium permanganate.

These tests do not characterize the barrier layer chemically beyond showing that it is highly resistant to heat, water, organic solvents, nonalkaline detergents and weak solutions of strong acids, but is readily disrupted by alkaline solutions and an oxidizing agent.

3. *Location of the electrolyte barrier.* Dusting the epicuticle surface of isolated cuticles with abrasive emery powder (120 mesh) and rubbing the powder around with a soft brush raises the conductance. Repeated or more vigorous rubbing with the brush raises the conductance further. Prolonged rubbing lowers the impedance to a very small value, and the change is irreversible. Greater abrasion is produced by rubbing with emery cloth. Such treatment, even though gently done, effectively eliminates the electrolyte barrier.

Thin serial sections prepared from the above cuticles show little microscopically detectable effect from the rubbing with emery powder even though the barrier may have been shown by an impedance measurement to have been almost abolished (Fig. 8). In some places in some sets of sections minute breaks can be located in the thin outer epicuticle, but no scratches extending deep into the inner epicuticle were found. Similarly, light rubbing with emery cloth produces no deep scratches though heavier rubbing does. This is most readily seen when the scratches are subsequently given contrast by applying ammoniacal silver nitrate to the surface (WIGGLESWORTH, 1945). Minute breaks in the outer epicuticle permit silver deposits to form in the cuticle. When such breaks were numerous the impedance of the membrane was found to be low. Deeper scratches extending well into or through the inner epicuticle are correlated with nearly complete elimination of the electrolyte barrier, as shown by near zero impedance values and by heavy silver deposits in both epicuticle and procuticle.

The above data indicate that the electrolyte barrier is distinct from the barrier to water shown by WOLFE (1955) and confirmed here, and also distinct from the layer responsible for the phenomenon called asymmetrical penetration (RICHARDS *et al.*, 1953). The data further indicate that the electrolyte barrier is the thin outer epicuticle to which no function has previously been assigned. This conclusion is consistent with the fact that the electrical data call for a barrier layer not more than a few hundred Ångström units thick, the abrasion data call for the layer to be at or exceedingly close to the outer surface, and the chemical data call for a layer resistant to organic solvents and to mild acid treatment but destroyed by mild alkali treatment.

One point requires further explanation. Thin sections stained with Mallory's triple stain do not show any microscopically detectable alterations of the epicuticle due to the treatments listed as not disrupting the electrolyte barrier. But sections confirm the report (DENNELL, 1946) that the inner epicuticle is completely dispersed by both acid and alkali, whereas the outer epicuticle is not dispersed by either. However, there is a real difference between the effects of acid and alkali treatments. The outer epicuticle is not detectably affected by mild acid treatment; it remains a

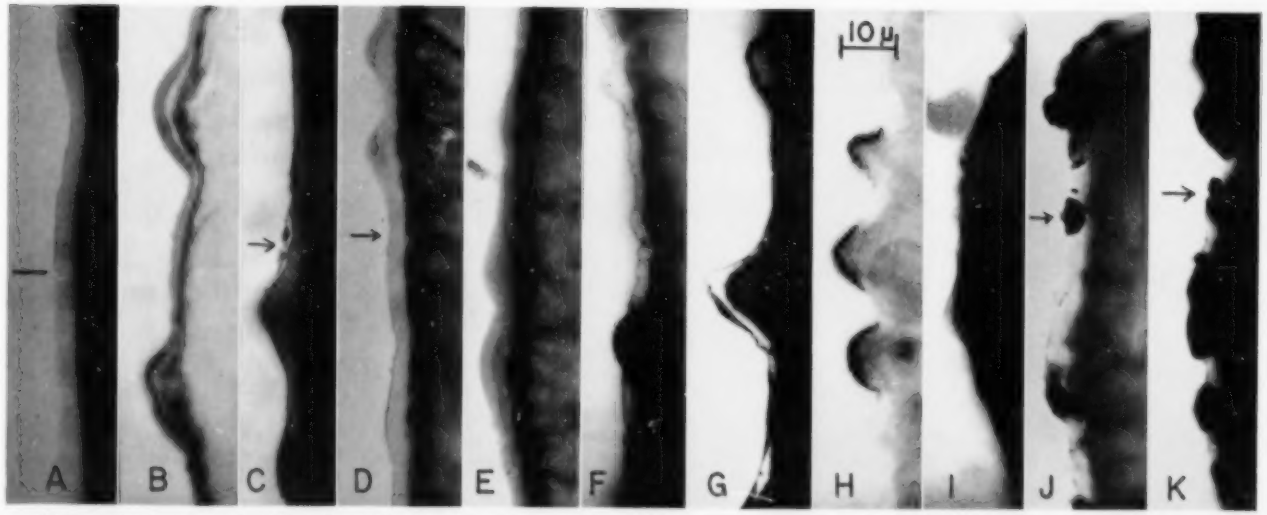

FIG. 8. Sections showing the epicuticle of last instar larvae of *Sarcophaga bullata* after various treatments. Mallory's triple stain, colour filters used to enhance contrast. Figs. A–I from thin sections (<1μ). All photographs at same magnification. (A) Section of a normal cuticle; outer epicuticle distinct above horizontal line, absent below this line (torn loose in sectioning). (B) Cuticle after impedance measurements and treatment with hot water and an organic solvent (this is membrane used for curves in Fig. 5B); outer epicuticle not distinct in photograph, inner epicuticle consisting of darker-staining outer band and light-staining inner zone central to which is the dark-staining outer edge of the procuticle. (C) Cuticle scratched with emery cloth and showing cut through outer epicuticle at place where it has raised away from the underlying inner epicuticle (arrow). (D) Another cuticle scratched with emery cloth showing at arrow the minuteness of scratches known in this case to have caused a reduction of over 95% in electrical impedance and resistance values. (E) Cuticle showing essentially normal appearing epicuticle after treatment with 0·1% NaOH at room temperature for 40 hours; this treatment caused the outer epicuticle to change to red-staining and reduced the electrical impedance value by about 98%. (F) Cuticle after treatment with simmering 1% NaOH for 5 minutes; most of the inner epicuticle is dispersed leaving the outer epicuticle plus adjacent zone of inner epicuticle as a contrastingly red line. (G) Another area from same cuticle photographed with a different colour filter. (H) Cuticle after treatment in simmering 10% NaOH for 15 minutes; epicuticle readily photographable only over spines where the outer zone of the inner epicuticle is still present, but under microscope the outer epicuticle is seen to be continuous. (I) Cuticle after treatment in simmering 10% NaOH for 6 hours; the inner epicuticle is completely gone but outer epicuticle is present even though faint-staining. (J) A 10μ section of an abraded cuticle treated with ammoniacal silver nitrate, and showing silver deposits from minute, small and deep cuts into the epicuticle. (K) Another photograph from the same cuticle showing the actual cut through the outer epicuticle permitting penetration with subsequent silver precipitation.

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continuous sheet and still stains blue with Mallory's stain. But the outer epicuticle after moderate treatment with hot alkaline solutions is altered so that it stains red with Mallory's stain. With prolonged treatment in hot alkali most of the red-staining material disappears leaving an exceedingly thin sheet that appears colourless but can be shown by the use of colour filters to be stained red rather than blue. The change in staining property of the outer epicuticle cannot be interpreted in chemical terms, but, in conjunction with the electrical and abrasion data, it leads to the conclusion that alkali destroys the ionic barrier properties of the outer epicuticle without destroying the layer itself.

DISCUSSION

Application of the parallel plate condenser equation to determination of barrier thickness requires knowledge both as to the area being measured and the dielectric constant of the material in the layer. It seems safe to say that the effective barrier is a flat sheet of material, and hence the area of the barrier equals the area of the piece of cuticle being measured, because neither sections examined with the light microscope nor isolated sheets examined in the electron microscope show indication of the outer epicuticle being a folded sheet. SANBORN and YOUNG's (1956) electron microscope pictures of thin cuticle sections also show no folding of epicuticular layers. But we can only guess at the dielectric constant (which fortunately does not have a large effect on the numerical value obtained from the equation). It seems reasonable to assume that the value will be in the range of wax and plastic values, *i.e.* somewhere between 2 and 10, perhaps near 6.*

The condenser equation is $C = kA/(4\pi d)$, where C = capacity in electrostatic units, k = the dielectric constant of the material, A = area in cm², and d = thickness in cm. Taking A as unity, the average capacity value of $0{\cdot}3\mu F/cm^2 = 270{,}000$ statfarads/cm². Solving with $k = 6$, the thickness is about 175 Å; with $k = 2{\cdot}7$, $d = 80$ Å; with $k = 10$, $d = 300$ Å. But the measurements show variation in the value of C (likely representing variation in thickness of the barrier). Recalculating with $k = 6$ and $C = 0{\cdot}14$–$0{\cdot}54\mu F/cm^2$, thickness extremes of 90 and 360 Å are obtained. Known variation in C and uncertainty as to the correct value for k, each introduce a variation of about a factor of 2 from the average value. Accordingly, as a first approximation, it seems best to say that the ionic barrier is 150 Å thick within a factor of 2 or 3. Some may prefer to say it is in the range of one to several hundred Ångström units thick.

Only one paper has been published dealing with the complex impedance of insect membranes. This is the old paper by COLE and JAHN (1937) on grasshopper egg membranes. Some similarities to the present data are apparent, but the differences from fly cuticles suggest that a different electrical model will be required.

* CHEFURKA and PEPPER (1955) record the dielectric constant of beeswax at 25° C. as 2·76, and other authors list similar values. But, if the barrier in these cuticles is such a free wax, one would expect removal by solvents we have found to have little effect on impedance. Accordingly, it seems preferable to select a value from the shellac-phenol-casein-urea series (range 4–7) rather than from the wax series (range 2–4).

The egg membranes do show a similar range of phase angles. The egg shell itself has a many times lower resistance which varies with the electrolyte concentration. But the embryonic membrane secreted by the serosa has a high resistance (the same magnitude as that of fly cuticles) which is reported as "probably independent" of the electrolyte concentration. This latter fact makes close comparison to our data undesirable, especially since recent advances in impedance analysis (*e.g.* SCHMITT's dynamic impedance method) indicate that these membranes are more complicated than just a resistance and a capacity.

When diffusion is the driving force and the cuticle is wet with water on both sides, no asymmetry has been discovered. In other words, rates when penetration is in the epicuticle→ endocuticle direction are the same as those obtained when the penetration is in the endocuticle → epicuticle direction. With isolated cuticles of fly larvae the data include heavy water, oxygen, KCl, and methylene blue (RICHARDS and FAN, 1949; RICHARDS *et al.*, 1953; and present data). Despite the differences between the cuticles of fly larvae and terrestrial insects, it seems probable that the same will be true for all insect cuticles.

However, when an insect cuticle is the membrane separating two halves of an asymmetrical system, an apparently asymmetrical penetration may be found, as first reported by HURST (see RICHARDS *et al.*, 1953). We have now found a second system in which insect cuticles (and certain other membranes) can behave as functionally asymmetrical membranes, and can, in fact, show several different kinds of asymmetry. If a relatively fast "electrical pumping" system is substituted for diffusion as the driving force, asymmetrical penetration results even though the system is symmetrical. Of more general interest is the fact that the insect cuticles in the dynamic impedance testing show unexpected properties of rectification and transient energy changes. These unexpected properties showing similarities between supposedly inert cuticles and supposedly vital or living nerve membranes have obvious implications but require further analysis.

It has been known for some years that the insect epicuticle is a heterogeneous assemblage of chemically different superimposed sublayers (see RICHARDS, 1951). One of these, the wax sublayer or wax impregnation, has been identified by WIGGLESWORTH (1945) as the effective barrier to water. No definite functions have been assigned to other sublayers although some speculations have been made. We now have data showing that there are at least three separable functions performed by different components of the epicuticle of fly larvae. These functions are summarized in the accompanying table.

The critical data from which this table is compiled are: (1) light surface abrasion raises ion penetration and probably oxygen penetration but has little effect on water penetration or the phenomenon of asymmetrical penetration; (2) deeper scratches accelerate ion penetration but have only moderate or little effect on water and the asymmetry phenomenon; (3) very deep scratches effectively eliminate the barriers to penetration of water, oxygen, and ions; (4) chloroform raises the rate of water penetration greatly but does not necessarily affect the rate of penetration of ions or oxygen or the asymmetry phenomenon; (5) mild treatment

SUMMARY OF KNOWN FUNCTIONS OF CUTICLE SUBDIVISIONS IN MAGGOTS

Outer epicuticle	Major barrier to ions Probably barrier to O_2	
Inner epicuticle		
(*a*) $CHCl_3$—soluble fraction	Water barrier	Lesser barrier to ions
(*b*) $CHCl_3$—insoluble fraction	Asymmetrical penetration (Perhaps O_2)	Lesser barrier to ions
Procuticle	Structural support No barrier to H_2O or H_2O solutes Perhaps a barrier to hydrophobic compounds	

with alkali raises the rate of ion penetration but has only a small effect on the rate of water penetration; and (6) electrical data show that the ion barrier must be exceedingly thin.

Conceivably the ion barrier is distinct from the oxygen barrier because the former is considerably modified by isolation of the cuticle whereas the latter is not.

The visible light compound microscope readily distinguishes between inner and outer epicuticles in fly larvae. Favourable sections show that the inner epicuticle can be further subdivided into (1) an inner zone which stains relatively lightly with fuchsin and which is readily dispersed by acid or alkali, and (2) a more densely staining outer zone which becomes denser staining and more resistant to dispersion near the outer epicuticle (Fig. 8b). By light microscopy, then, there are at least three sublayers recognizable which correspond in position to the three separable barriers treated above. Since the above work was finished, I have had the privilege of seeing a manuscript by SANBORN and YOUNG (1956) in which, on the basis of electron micrographs of sections of housefly larval cuticles treated with phosphotungstic acid, the epicuticle of a closely related species is shown to be divisible into four superimposed sublayers. A full analysis of the epicuticle remains to be done, but clearly a multiplicity of superimposed sublayers is indicated by both light and electron microscopy.

Other groups of insects are known to have complex epicuticles (WIGGLESWORTH, 1947), but the identity of the different subdivisions seems clearly not the same in all groups (DENNELL and MALEK, 1953). Thus, the latter authors compare the "inner epicuticle" of fly larvae with the "cuticulin layer" of *Rhodnius*, etc. Yet, although the cuticulin layer is only one of the four sublayers listed for *Rhodnius* cuticles, it is the one subdivided in fly larvae. But despite this apparent histological diversity in different groups of insects, impedance measurements clearly show that the component of the epicuticle which constitutes the electrolyte barrier is different from that which constitutes the water barrier (*Sarcophaga*, *Blaberus*, *Galleria*, *Corydalus*). It seems that both of the above will be found distinct from the sublayer giving the phenomenon of asymmetrical penetration, although conclusive data are available only for *Sarcophaga*. We are therefore led to conclude that one of the

reasons for the complex superposition of sublayers in the cuticle is that different sublayers are barriers to different groups of chemicals.

SUMMARY

1. Isolated cuticles of fly larvae (*Sarcophaga bullata*) measured in a polarographic cell give oxygen penetration values closely similar to ones reported by other authors from measurements of cutaneous respiration. It follows that the cuticle itself is the effective barrier to oxygen. This barrier is not affected by extraction with hot chloroform but is broken by surface abrasion.

2. Similar cuticles measured by complex impedance show that the principal barrier to electrolytes is the outer epicuticle which has a calculated thickness of the order of a few hundred Ångström units. This barrier is not broken by hot water, hot organic solvents, or dilute mineral acids, but is readily eliminated by surface abrasion, by oxidizing agents, and by alkaline solutions.

3. Comparison of these data with previously published data show that the heterogeneous epicuticle of fly larvae contains at least three recognizably distinct components which are respectively and separately the barrier to ions (and perhaps to oxygen), the barrier to water, and the site of the phenomenon called asymmetrical penetration (see Table). The ionic barrier is the outer epicuticle; the other two properties are located in the inner epicuticle, and appear to be due to the outer and inner portions respectively.

4. A few observations on other species suggest that in general the electrolyte barrier will be found to be distinct from the water barrier in insect cuticles.

5. Isolated cuticles of *Sarcophaga* larvae were found to have a capacitance of $0{\cdot}300 \pm 0{\cdot}0124 \mu F/cm^2$, a phase angle of $82{\cdot}6 \pm 1{\cdot}2°$, a more variable but always low conductance having a mean zero frequency resistance value of $38{,}000 \pm 3000$ ohms/cm², and a low "characteristic frequency", when the membrane is in 0·19 M KCl solution.

6. Of the above electrical properties, only the capacitance value and the phase angle remain reasonably constant. Treatments involving sorption of KCl or hydration can greatly increase the ionic permeability, but the increase is largely reversible. This provides an explanation for the apparent anomaly that the entire integument is more permeable to ions than an isolated cuticle.

7. Measurement of other electrical properties of isolated cuticles by a new method shows that these cuticles also exhibit rectification, polarization effects, and even bimodal polarization peaks which imply transient energy changes.

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ANTIFUNGAL ACTION OF CUTICULAR LIPIDS IN INSECTS*

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Abstract—Larvae of the silkworm (*Bombyx mori* L.) and the rice stem-borer (*Chilo simplex* Butl.) become highly susceptible to muscardines when their cuticular lipids are either mechanically or chemically removed. Lipids contained in the shed cuticles of silkworms have either a fungicidal or fungistatic action, according to concentration, on cultures of *Aspergillus flavus*. Three species of fungi grew better on aqueous extracts of shed cuticles from which the lipid constituents were removed than on aqueous extracts which contained the lipids. The most active antifungal constituents of cuticular lipids were free medium-chain saturated fatty acids, presumably caprylic acid or capric acid, which occur naturally in the cuticle. The antifungal activity of lipids extracted from the living cuticle was slightly greater than that of those extracted from shed cuticles.

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INTRODUCTION

EXCEPT for the work in immunochemistry, the disease-defence mechanism of the animal body has hitherto been relatively little studied from the viewpoint of so-called "antibiotic action" when compared with the same mechanism of the micro-organisms and the higher plants. The recent review by PAVAN (1952) lists only a few works on worms, mites, insects, and other invertebrates. Incidental to a study on liquid permeability, it was found that insect larvae whose outer surfaces had been treated to remove most of the epicuticular lipids were commonly attacked by pathogenic fungi. Study of this phenomenon has now shown that the epicuticular lipids of some insects can inhibit fungal development, and that the effect seems due mainly to the presence of free fatty acids of medium or short chain length.

REMOVAL OF EPICUTICULAR LIPIDS AND FUNGOUS INFECTION

During an investigation of the rôle of epicuticular lipids in the permeability of liquids, it was unexpectedly found that the caterpillars of the common rice stem-borer (*Chilo simplex* Butl.) were severely attacked by a kind of pathogenic fungus (*Isaria farinosa*?) when their epicuticular lipid was removed by rubbing the body surface with fine alumina dust. Larvae not so treated were not attacked. Both treated and untreated larvae were kept in their normal habitat in the moist stems of rice plants.

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A large number of larvae of *Chilo simplex* (in May) and *Bombyx mori* (in July) were deprived of their surface lipids either mechanically by rubbing with fine alumina dust for 10 minutes ("rubbed" in Table 1) or chemically by wiping gently with carbon tetrachloride ("wiped" in Table 1). Both species were then sprayed with spores suspended in water of the pathogenic fungi, *Aspergillus flavus* and *Beauveria bassiana*, and the larvae were placed individually in glass vials, the temperature and moisture of which were maintained at 26–28° C. and 95% R.H. The number of the larvae dying of the fungous diseases after treatment is indicated in Table 1.

TABLE 1—Percentage of infection by *Aspergillus flavus* and *Beauveria bassiana* and average number of days of incubation period of disease of larvae of *Chilo simplex* and *Bombyx mori* after removal of cuticular lipids by rubbing with alumina dust (*R*) and by wiping with CCl_4 (*W*) and of nontreated individuals (*C*).

Insects and instar	*Chilo simplex*					*Bombyx mori*							
						III		IV		V		Mature	
Fungi	*Aspergillus*			*Beauveria*		*Aspergillus*							
Treatment	*C*	*R*	*W*	*C*	*R*	*C*	*W*	*C*	*W*	*C*	*W*	*C*	*W*
% of infection	23	67	100	35	75	70	100	75	100	78	100	87	100
Incubation period	14·3	13·0	6·9	14·6	11·0	9·5	8·0	10·7	6·8	10·9	7·0	12·6	7·0

It may be suspected that such treatments as described above, especially surface abrasion, will result in mechanical injuries to the cuticle that may facilitate fungous invasion. However, it was possible to show by microscopical examination and by means of the argentaffin reaction that there were no visible injuries. Nevertheless, in all instances the total number of infected larvae and the speed of development of the attack of both fungi were greater in the treated larvae than in the controls. It would therefore seem that a layer of free lipids on the surface of the cuticle restricts the invasion of pathogenic fungi through the skin, in addition to waterproofing the skin.

The mechanism of this defence may be either physical or chemical. Although it is very probable that the epicuticular lipid serves essentially as a mechanical barrier to prevent many micro-organisms from reaching the inner layers of the cuticle, it is also possible that the epicuticular lipids possess chemical properties that increase the resistance of the cuticle against microbial invasion, so that one may say, for instance, insects produce antifungal substances of lipid nature.

PRELIMINARY EXPERIMENTS

The exuviae of insects appear to be the most suitable materials from which the lipids can be obtained because they are free from contaminations of any internal tissues and consist of pure cuticle. They are composed of epicuticle in

the complete condition and of exo- and endocuticle, partly changed by the moulting process. As, at present, some confusion exists in the literature as to whether the exocuticle is impregnated with lipids or not (DENNELL and MALEK, 1955), the lipid substances extracted from the exuviae are here called "cuticular lipids" instead of "epicuticular lipids".

As a preliminary experiment on the action against the development of a pathogenic fungus, *Aspergillus flavus*, three kinds of materials prepared from an ether extract, a cold water extract, and an ether extract of steam distillate of carefully stored silkworm exuviae of the 4th moult were compared with one another. Each extract, with the exception of the aqueous one, was dissolved in acetone in the proportion of 1 : 100, and this acetone solution was mixed with Dox modification of Czapek solution agar soon before the coagulation of the agar in a concentration of 1 : 5 so as to make a 500-fold dilution of the original substances. Acetone was used as solvent on the ground that it dissolves the extracts very well and can be mixed with aqueous culture media in any desired concentrations. The aqueous extract was added directly to the medium by dissolving it in water.

The nutrient agar containing active substances was poured into each of three small cylindrical glass vials (diameter 14 mm, height 2·8 mm) placed at equal distances on a slide glass until about eight-tenths of the depth of the ring was filled. Three vials contained the single result of one dilution of the active substances. The results indicated in the tables are average values. After the medium got completely cold, one loopful of aqueous suspension of the fungous spores (2 loopfuls of spores in 1 cm. distilled water) was placed on the centre of the surface of the medium. The slides with vials were placed in a loosely lidded Petri dish lined with moist filter paper. They were kept in an incubator at 27–28° C. As the Czapek–Dox medium was nearly transparent to a depth of about 2·3 mm, it could be poured into cylindrical vials where every phase of the growth of the inoculated fungi could be clearly examined under the microscope. Thus the germination of spores, elongation of hyphae, diameter of colonies, and formation of new conidia were noted at regular (mostly 24-hour) intervals following the inoculation.

In addition to the "normal control" consisting of the normal Czapek–Dox medium without acetone or the extracted substances added, the "acetone-control" was prepared. It consisted of 1 part acetone plus 4 parts culture medium and corresponded to the quantity of acetone contained in the 1 : 500 dilution of the extracted substances. If the dish is covered closely so that the added acetone is prevented from evaporating, the inhibitory action of the acetone on the development of fungi is rather great; however, if the dish is covered loosely as was done throughout the experiments, the acetone soon evaporates and its inhibitory action is negligible.

It was clearly shown that the ether extract of the silkworm exuviae inhibits the growth of the fungus. For instance, 24 hours after the inoculation the percentage of spores germinated is smaller and the hyphae, if they grow, are not so great in length as to form a mycelial colony (much less to produce new conidia) in the medium to which the ethereal extract is added as compared with the acetone

medium. The preventive action was proved to be far greater in the medium to which the ether extract of the steam distillate of the exuviae was added. In this medium not a single spore germinated even after 72 hours. It was an unexpected fact that the pure aqueous extract of the exuviae evidently possesses an inhibitory action, though it is not so strong as that of the other two extracts.

If, as described above, the substances obtained from the exuviae on ether-extraction exhibit the growth-retarding effect on fungi *in vitro*, it is to be expected that the exuvial substances from which the ether-extractable components are removed will culture the fungi better than those of normal exuviae containing them. Ten grammes of last instar larval exuviae of silkworms from which the lipid constituents had been completely removed by extraction with ether for 30 hours were extracted with cold distilled water for 3 hours. The extract was filtered to remove the residues. Using this filtrate (instead of using distilled water) the Czapek–Dox solution agar was prepared, and the spores of three species of fungi, *Aspergillus flavus*, *A. clavatus*, and *Mucor* sp., were inoculated on it in order to compare their growth with that on the same amount of the filtrate derived in the same manner from the aqueous extraction of the normal exuviae containing lipids. The production of new spores on the mycelia occurred as follows. No spores were formed within 17 hours in *A. flavus* and *A. clavatus* and within 24 hours in *Mucor* sp. in the media supplied with the aqueous extracts of the exuviae with the lipid constituents, in contrast to the state of limited spore production (+) in the media without lipids (Table 2). Great numbers of white spores (++) from the three species were not obtained from media supplied with aqueous extracts plus lipid constituents until 31, >72, and 48 hours, respectively, had elapsed. Under these conditions maximum numbers of yellow spores (+++) were not formed until 72, >72, and 72 hours had elapsed. The corresponding states in the media without lipids were 24–31, 48, and 31 hours (*i.e.* ++) and 31, 72, and 72 hours (+++), respectively. The result shows that the lipid-extracted exuviae accelerate the growth of the tested micro-organisms as compared with those which contain lipids.

It was thus clearly proved that the insect cuticle contains substances capable of chemically preventing the growth of certain fungi, and it was assumed that the substances might be of lipid nature soluble in ether, partly soluble in water, and volatile on steam-distillation.

POSSIBLE COMPONENT MAINLY RESPONSIBLE FOR ANTIFUNGAL ACTION OF CUTICULAR LIPIDS

Seven kinds of fraction products, *A* to *G*, were obtained on extraction with ether or cold water or with steam distillation, singly or by combination from the air-dried exuviae of the last larval moult of the silkworm by the method shown in Fig. 1. All the products were dried, dissolved in acetone, mixed with the Czapek–Dox solution agar, and a loopful of spores of *A. flavus* was seeded on it as described in the preliminary experiment. Growth was compared in an incubator at 27–28° C and 95% R.H. The results are recorded in Table 2.

TABLE 2—Antifungal action of various extracted products from the exuviae of the silkworm against *Aspergillus flavus* at 1 : 500 and 1 : 1000 dilutions: (*a*) approximate percentage of spores germinated determined by visual count; (*b*) length of hyphae in mm; (*c*) diameter of mycelial colony in mm; (*d*) new spore formation on the mycelia; (−) no spores formed; (+) only very few spores formed (as many as may be counted in the microscopic field), and all the spores are white in colour; (++) spores formed, but the number is too great to be counted; their colour is the same as in "+"; (+++) the microscopic field is completely full of spores of the yellow colour characteristic of this fungus.

Fraction	Dilution	24 hr			48 hr				72 hr			Order
		a	*b*	*d*	*a*	*b*	*c*	*d*	*a*	*c*(*b*)	*d*	
A	500	100	0·49	−			6	++		8	++	5
	1000	100	0·67	−			7	++		10	+++	
B	500	0			100	0·66		−		4	++	3
	1000	40	0·06	−	100	1·23		+		6	+++	
C	500	80	0·26	−	100		3·5	++		9	++	4
	1000	100	0·62	−			9	++		10	+++	
D	500	0			0				0			1
	1000	0			80	0·23		−	100	5	+	
E	500	100	0·49	−			8	++			+++	6
	1000	100	0·57	−			9	++		10	+++	
F	500	0			50	0·10		−	100	(0·67)	−	2
	1000	0			100	0·82		−		6	++	
G	500	100	0·57	−			9	++		10	+++	7
	1000	100	0·66	−			9	++		10	+++	
Acetone control	—	100	0·95	−			10	++		10	+++	

All fractions retarded the development of the fungus, but in different degrees. Judging from all criteria of growth, the order of the retarding action is :

$$D>F>B>C>A>E>G.$$

The retarding action of both *D* and *F* fractions is far stronger than that of any other fractions. Compared with these, the actions of both *B* and *C* are far weaker, though they greatly surpass those of *A*, *E*, and *G*.

What chemical substance does each fraction represent? BERGMANN (1938) demonstrated that the ether extracts of silkworm exuviae contain two major components, namely, a mixture of paraffins of the probable order C_{27}–C_{31} and a mixture of the esters of alcohols and acids of the probable order of C_{26}–C_{30}, but he left untouched substances as free fatty acids or volatile substances considered to have a close relation to antibiotic activity. Fortunately SHIKATA (1954), one of our collaborators, has recently published his results of detailed chemical

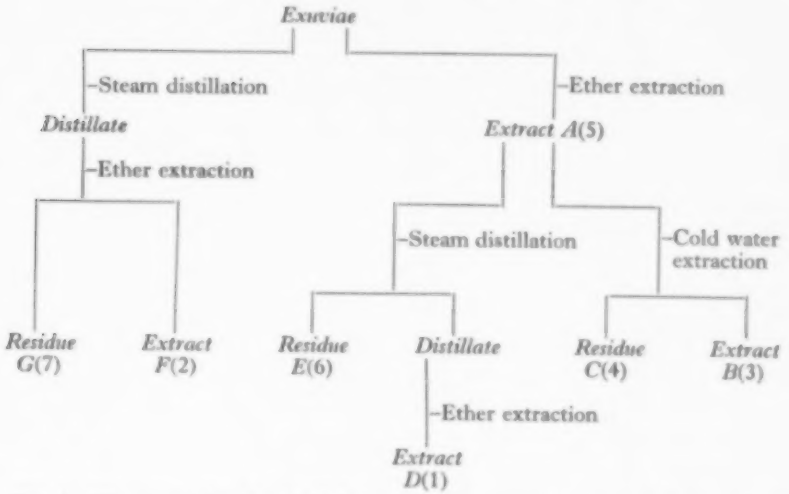

FIG. 1. The steps taken for obtaining various components of exuvial lipids for comparison of their antifungal action (see Table 2). The numbers in parentheses denote the order of the strength of the action of each substance.

analysis of lipids contained in the silkworm exuviae of the last larval moult. According to him, the exuviae consist of the following components: (*a*) Small amounts of glycerides. (*b*) Water-soluble but nonvolatile acids such as succinic and an unknown acid higher than adipic. (*c*) Small amounts of volatile fatty acids existing in the free state in the cuticle that can be identified as caprylic (C_8) or capric (C_{10}) acid. (*d*) Crude wax consisting of: free fatty acids, unsaturated (unidentified) and saturated of longer chain length than melissic acid (C_{30}), ester-forming acids composed of the same ones as in the free acids, and a large quantity of unsaponifiable matter composed of a higher alcohol considered to be higher than myricyl and a hydrocarbon assumed to be either unsaturated or saturated and higher than triacontane.

Since both of the *D* and *F* fractions in our Fig. 1 and Table 2 are volatile on steam distillation and ether-soluble, they are believed to be composed mainly of the free volatile fatty acids reported in SHIKATA's data. Based on the neutralization value (as high as 121·5), it is evident that the free acids contained in the crude wax correspond neither to *D* nor *F*. Fraction *B* is thought to be composed of water-soluble, nonvolatile acids mixed with water-soluble, volatile ones. Fraction *C* and *E* may contain wax-ester, glycerides, and paraffins as a principal part. All substances soluble in ether are of course represented by *A*. Fraction *G* is supposed to contain volatile substances other than acids. Thus, on the whole, the fractions that possess remarkably strong antimicrobial activity are believed to contain fatty acids, either volatile (*D* and *F*) or water-soluble (*B*), as their main constituent, while weaker actions are shown by fractions (*C*, *E*, *G*) that consist mainly of wax-ester, glycerides, paraffins, and ether-insoluble volatile substances.

As SHIKATA's volatile acids, presumably caprylic or capric acid, as well as our volatile substances, were obtained on steam distillation or ether extracts or of exuviae and not by adding to them any acid or alkali nor by superheating, it appears that they do not come out as a result of hydrolysis of their esters during the course of the distillation but occur naturally in the free state in the cuticle lipid. It must be noted here that the free acids could not have originated from the so-called rancidity of lipids because special precautions were always taken to avoid the hydrolytic changes of cuticular lipids catalysed by moisture, light, and heat. These precautions consisted of keeping the materials in desiccators containing $CaCl_2$ and placed in a dark room at low temperatures. The materials were used for experiment, usually within 1 or 2 months following moulting. With regard to this point, some comparisons were made on the changes of antifungal strength with exuviae of the last larval moult which were preserved in a paper bag left in an ordinary laboratory room and with exuviae in a desiccator placed in a dark cool room. It was found that the antifungal action does not change essentially, at least during the first 6 months when the material is stored in the desiccator, whereas it increases gradually when left unprotected in the laboratory.

As for the free unsaturated fatty acids (unidentified) detected as components of the crude wax by SHIKATA, it is considered that they may not play an important rôle in the inhibition of the development of at least *A. flavus*, for all of the fractions supposed to contain these acids never show a conspicuous toxicity when tested in the equal concentration.

Thus it is highly probable that the substance or substances mainly responsible for the antifungal action of the cuticular lipids of insect exuviae, and presumably also in the living integument, are volatile (on steam distillation), water-soluble, saturated fatty acids, similar to the silkworm skin acids of medium-chain length, such as caprylic (C_8) or capric acid (C_{10}), that occur in the free state.

STAGES OF DEVELOPMENT OF FUNGI AFFECTED

(*a*) *Effect on spore germination*

The fact that the germination of spores is greatly affected by the active lipids will be referred to at length in subsequent papers. However, the problem of whether the so-called antifungal action of the lipids is fungicidal or fungistatic may now be considered. Spores of *A. flavus* left ungerminated in the liquid medium of Czapek mixed with the ether extract of the steam distillate of silkworm exuviae from the 4th moult* were seeded on the normal medium without the active principle, and the power of germination was examined. It was found that spores subjected to both 1 : 500 and 1 : 1000 toxic dilutions for 72–96 hours did not germinate at all when returned to the normal medium. On the other hand, the germination power is retained in the spores whose sojourn in the toxic media at the weak concentrations of 1 : 2000 or more is as short as 24 hours,

* The action of cuticular lipids contained in the exuviae from the 4th larval moult is far stronger than that from the final moult: no spores germinated until 96 hours after inoculation even in a concentration as weak as 1: 4000.

or even so long as 96 hours. Thus the action of the cuticular lipids on fungi may be divided into fungicidal and fungistatic ones according to the concentration *in vitro* and the duration of action upon the micro-organisms.

(*b*) *Effect on hyphal elongation*

One loopful of Czapek liquid medium containing a large number of hyphae of *A. flavus* which had grown just to the length of about 82–164 μ after culture for 16 hours, at 25° C was inoculated in both Czapek liquid and Czapek–Dox agar medium supplied with the ether extract of steam distillate of the exuviae from the 4th moult. The elongation growth of the hyphae did not take place in either media in the concentrations of 1 : 500 and 1 : 1000. They grew with or without production of new conidia only in concentrations weaker than 1 : 2000. From these results it is certain that the extracted substance has an influence also upon the hyphal growth of fungi.

(*c*) *Effect of spore formation*

Hyphae of *A. flavus* about 1 mm in length and in the stage immediately prior to spore production obtained from culture in the liquid medium maintained at 30° C for 20 hours, did not yield any new conidia during 24–48 hours at 27–28° C on putting them into toxic media of concentrations from 1 : 500 to 1 : 2000. A small number was produced in the concentration of 1 : 4000.

It was proved in this way that the active components of the cuticular lipid inhibit not merely the germination of spores but also the elongation of hyphae, the growth of the colony, and the production of spores of fungi.

COMPARISON BETWEEN EXUVIAE AND LIVING INTEGUMENT

Information so far mentioned on the antifungal actions of cuticular lipids was obtained exclusively from the shed skins of the silkworm.

TABLE 3—Comparison of antifungal actions of lipids from living integument and exuviae of mature silkworm against *Aspergillus flavus*.

From	Dilution	24 hr			48 hr			72 hr		
		a	*b*	*d*	*a*	*b*(*c*)	*d*	*a*	*c*	*d*
Living integument	500	0			0			0		
	1000	10	0·02	—	50	0·48	—	100	9	++
	2000	50	0·20	—	100	(7)	+		15	+++
	4000	40	0·20	—	100	(6)	+		16	+++
	A-contr.	80	1·20	—	100	(13)	++		15	+++
Exuviae	500	0			30	0·13	—	50	4	+
	1000	20	0·06	—	100	0·84	+		10	++
	2000	80	0·84	—	100	(10)	++		15	+++
	4000	100	1·20	—		(13)	++		15	+++
	A-contr.	100	1·36	—		(13)	++		15	+++

Living cuticle, presumably stripped of epidermal cells, was prepared by removing it from the body of mature silkworms by surgery under carbon dioxide anaesthesia and then by freeing it from adhering tissues as completely as possible by carefully scraping its innerside with the smoothed edge of a microscope slide. After complete absorption of blood on the cuticle with blotting paper, the material was placed immediately into a flask for steam distillation. Exuviae of the last larval moult were collected from the cocoons which contained the pupae that were transformed from the larvae of the same source. The antifungal actions of the ether extract of the steam distillate of both materials are compared with one another in the results shown in Table 3.

It may be seen from the table that the exuvial lipid shows an antifungal activity almost similar to that of living cuticle, though its strength is weaker in some degree. Thus it may not be unreasonable to discuss the antibiotic action of the living integument in terms of the information obtained from the exuviae.

DISCUSSION

The intact integument, or exoskeleton of insects, is a particularly effective barrier to invading micro-organisms, and this effect has been thought to be dependent chiefly upon its mechanical structure: a thick, nonliving, cuticular wall provided on the surface with a thin, physically impervious layer of epicuticle. However, it may not be unreasonable to suppose that this mechanical effect is supplemented in other ways. Experimental evidence that the integument of insects contain lipids capable of restricting the growth of pathogenic fungi *in vitro* is given here. But whether the insect body really makes use of its natural lipids or fatty acids occurring in the cuticle in preventing invading fungi may not be stated with certainty so far as the present experiments conducted *in vitro* are concerned. Judging, however, from the following fact, which will be published in detail in the next paper, this is probably true also *in vivo*. The antifungal action of lipids or of free fatty acids obtained from the silkworm integument, either of races or at stages of development that had been proved most resistant to muscardine from both rearing experiences and the infection tests, was proved to be stronger than that of the substances from susceptible races or stages.

Similar facts are also found in the literature where experimental evidence is given that the lipids or the fatty acids play a part in the defence against infections of human skin and hair (BURTENSHAW, 1942; ROTHMAN *et al.*, 1946; WEITKAMP *et al.*, 1947; MACKENNA *et al.*, 1950).

It appears that no general agreement has been reached with regard to the question of which fatty acid, saturated or unsaturated, acts as a potent agent for the inhibition of bacterial growth. Claims that unsaturated acids like oleic, linoleic, and linolenic acid exert a striking toxic effect on a number of bacteria in a definite concentration (POLLOCK, 1949; KODICEK, 1949) contrast with the views that the powerful depressing effect is represented chiefly by saturated acids of medium or short chain length (SPOER *et al.*, 1949).

Studies on the inhibitory action against fungi are scanty as compared with those on bacteria. In general, fungi, like other non-bacterial organisms, do not seem to succumb to the action of fatty acids as readily as bacteria. Most of the published studies indicate that against fungi it is the saturated fatty acids of shorter chain length, volatile and water-soluble, which are, in general, by far the most active. They are, for example, caprylic acid (*Penicillium palitans* and *Oidium lactis*, STOKOL, 1928), propionic, capric, and caprylic acid (*Aspergillus niger* and *Trichophyton interdigitale*, WYSS *et al.*, 1945), and propionic and caprylic acid (*Trichophyton gypseum*, FOLEY *et al.*, 1947). ROTHMAN *et al.* (1945, 1946) confirmed that the active principles of human hair lipids affecting the growth of *Microsporon audouini*, one of the causal fungi of ringworm, are saturated acids of C_7–C_{11} series. This finding contrasts with that of BURTENSHAW (1942), who stated that the bactericidal action of human skin is chiefly dependent upon the constituent unsaturated acids. KOIDSUMI and NAKAMURA have obtained ethereal extracts from the small intestine of the pig to compare the anti-fungal action of each component on the growth of some fungi (*Aspergillus flavus*, *A. clavatus*, and *Mucor* sp.) and have demonstrated that (1) the action is strongest in the component of free, short-chain, saturated acids composed mainly of butyric acid, (2) that the fraction of unsaturated acids, the chief component of which is oleic acid, shows a very weak action, and (3) that practically no inhibition is obtainable from the mixture of higher saturated acids such as myristic, palmitic, and stearic acid, when the comparison is made with each fraction in an equal concentration. The results will be published elsewhere in the near future.

As to the relation between the number of carbon atoms of saturated monobasic acids and their germicidal or other biological action, numerous reports have appeared in the literature showing good examples of the variation of biological activity within members of homologous series. The usual effect hitherto found by many authors in this homologous series is a gradual rise to maximum activity at a certain chain length, *i.e.* mostly at C_6–C_{12}, followed by a fall as the series is ascended further. Sometimes the peak may be as low as 1 or 2 carbon atoms. Thus the maximum activity is attained on *Staphylococcus aureus* in the soaps of the series of C_6–C_8 acids (EGGERTH, 1926; COWLES, 1941) or those of C_9–C_{11} acids upon tubercle bacillus (KÜSTER and WAGNER-JAUREGG, 1944), and on tubercle and other acid-fast bacteria in formic and capric acid in the series of acids of short or medium chain length respectively (IIJIMA, 1935). PAVAN (1952) attributed the antibiotic principle of ant toxin of some Formicids to formic acid. According to an investigation by FRANKE and SCHILLINGER (1943), the respiration of some acid-fast bacteria is depressed most markedly by the action of acids of C_{10}–C_{14} series. SPOER *et al.* (1949) made an extensive study of the antibacterial actions of fatty acids obtained from extractions of *Chlorella* cultures as well as of tissues of some higher plants. They failed to prove the existence of toxic action by the unsaturated acids on *Staphylococcus aureus*. But saturated acids produced by photo-oxidation of the unsaturated acids have an effect; the C_{11} compound especially shows a high toxicity. SPOER *et al.* (1949) further examined the pH,

solubility, diffusibility, and other physico-chemical properties of saturated acids in pure form with regard to the anti-bacterial action and found capric and lauric acid to be the most powerful agents viewed from the criterion of intrinsic toxicity as well as solubility.

Thus the results of the experiments recorded in this paper showing that the growth of pathogenic fungi is inhibited by ethereal extracts of the integument as well as of exuviae of the silkworm, and that the active principles mainly responsible for this lipid action may be free saturated fatty acids of medium chain length such as caprylic or capric acid, are in good agreement with the results found in a survey of the literature.

It must be noted, however, that although it seems that the above-mentioned saturated acids are the most active antifungal constituents of the cuticle lipids, it does not necessarily follow that they play a principal rôle in the action *in vivo*, because the total toxicity of any substance depends, in general, not merely upon its intensity but also upon its amount, *i.e.* the amount occurring in the cuticle. Owing to the technical difficulties in the quantitative determination of each active component, this problem has not yet been solved. But, from the results of investigations now being prepared for publication, it would seem that lipids have an important antifungal action—*in vivo*. The results show that there is a close parallelism between the susceptible races and stages of development of the silkworm and the antifungal action of the ether extract of the steam distillate of the cuticle of the same races and stages.

The conclusion may therefore be drawn that the barrier imposed by the exoskeleton of insects against the invasion of pathogenic fungi due to its mechanical structure may be increased further by an antifungal action in the chemical sense, that is, by the action of lipids, mainly medium-chain saturated fatty acids existing in the free state in the cuticle.

SUMMARY

1. The larvae of silkworms and rice stem-borers become highly susceptible to muscardines if their cuticular lipids are removed either mechanically or chemically.

2. The lipids contained in the exuviae of silkworms show a definitely antifungal action *in vitro* against the growth of a pathogenic fungus, *Aspergillus flavus*.

3. The aqueous extract of the exuviae from which the lipid constituents were removed cultured three species of fungi, including *Aspergillus flavus*, better than the extract of normal exuviae which contained the lipids.

4. Of all the constituents of exuvial lipids, free medium-chain saturated fatty acids, presumably caprylic or capric acid, not formed by the rancidity of the lipids but existing naturally in the cuticle, are by far the most active.

5. The fungus is affected by the lipid action throughout its growing period. The germination of spores, the elongation of hyphae, the size of the colony, and the formation of conidia are all affected.

6. The antifungal action is either fungicidal or fungistatic according to the concentration and the duration of action of the active constituents.

7. Exuvial lipid shows an almost similar antifungal activity to that of living cuticle, although its strength is somewhat less.

8. It is highly probable that the function of the insect exoskeleton as a barrier against the invasion of pathogenic fungi due to its mechanical structure is increased further by an antifungal action of free saturated fatty acids of medium chain length existing freely in the cuticle.

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THE INFLUENCE OF FLIGHT NOISE ON BEHAVIOUR IN THE DESERT LOCUST *SCHISTOCERCA GREGARIA* (FORSK.)

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Abstract—The paper describes experiments carried out in Africa to investigate the possibility that the flight noise of the Desert Locust influences the gregarious behaviour observed in swarms and plays a part in such behaviour as mass take-off. The sensitivity of the tympanal organs to pure tones in the range 0–20 kc/s was determined, and the methods used to record and analyse the sounds produced by single tethered flying locusts and also swarms of locusts in the field are described. It was found that under certain conditions a single flying locust emitted a noise due to impact between the wings and hind legs; this noise is termed "the wing-beat noise" and occurs at the frequency of the wing beat, that is at between 17–20 c/s. This noise apparently does not occur during level flight but appears during landing and take-off, when it may have behavioural significance. Behaviour experiments involving playback of recorded flight noise to locust swarms and the release of deafened locusts into settled and flying swarms are described. From the data on tympanal sensitivity and the acoustic power generated by portions of flying swarms it was possible to form estimates of the distances over which locusts can hear the flight noise of other locusts and of swarms.

The principal behaviour patterns of flying swarms are discussed in relation to the data derived from the acoustic and behaviour experiments.

INTRODUCTION

THE behaviour of swarms of adults of *Schistocerca gregaria* in the field in Africa has been described by GUNN (1948), KENNEDY (1951), WALOFF and RAINEY (1951), and RAINEY and WALOFF (1951).

The outstanding feature of these vast aggregations of locusts is their ability to cover long distances (hundreds of miles) in coherent groups. The drop-out of locusts over the path traversed by a swarm is considerably less than the scatter to be expected from an aggregation which is constantly under the influence of forces tending to disperse it, such as wind, convective air movements and effectively random flight orientation in the swarm (RAINEY and SAYER, 1953; RAINEY, in preparation). Furthermore, behaviour patterns exist which enable the swarm to move off from a roosting site together, despite the fact that a state of readiness for flight is reached by different individuals at different times, and that "milling" flights round the roosting site by groups of locusts (KENNEDY, 1951) can and do take place for some time before the mass movement of the swarm begins.

The above considerations have led various workers to postulate the existence of a "cohesive factor" in swarms by means of which individuals remain in communication with one another or with a group and with the swarm as a whole, and

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so combat the effect of the dispersive forces acting upon the latter. Such a "cohesive factor" must operate through the sensory receptors of individuals, and the organs which possess the necessary range of action for this can only be visual, auditory, or olfactory.

Little or nothing is known of the olfactory receptors of the Desert Locust or of the part they play in the gregarious behaviour of the insect, although NORRIS (1954) has shown that olfactory stimulation has an effect on sexual maturation and reproduction in cage-bred locusts. It is doubtful, however, if olfactory stimuli would be sufficiently powerful to operate in the confused atmospheric conditions in which swarms often migrate.

Visual and auditory receptors have directional characteristics and can operate over fair distances: this may enable them to act as mediators of a "cohesive factor", and this paper presents the results of a preliminary investigation into the auditory behaviour of the Desert Locust, with particular reference to its behaviour in swarms.

PRELIMINARY LABORATORY EXPERIMENTS

If auditory reactions bring about a cohesive effect in swarms the only source of sound stimuli from flying locusts is the flight noise, and the auditory receptors which are likely to be involved in receiving such stimuli are the paired tympanal organs. The suggestion that some such mechanism might be involved in swarm behaviour has been previously made by PUMPHREY (KENNEDY, 1951), who made a preliminary physiological investigation of the capability of the tympanal organs of *Locusta* and *Schistocerca* of detecting the flight noise of flying locusts. The details of these experiments have never been published. The following preliminary experiments were therefore carried out:

(*a*) Investigation of the variation of threshold intensity with frequency in the tympanal organs.
(*b*) Investigations on the reception of flight noise by the tympanal organs.
(*c*) A preliminary analysis of the frequency spectrum and intensity of the wing noise of a single locust.

These experiments were carried out at the Imperial College Field Station with locusts taken from the insectary there; these locusts were adult but immature, as in many migrating swarms, including those studied during the present work.

The determination of the variation of threshold pressure with frequency in *Schistocerca* was made in a similar manner to that of PUMPHREY and RAWDON-SMITH (1936a) for *Locusta*, except that a sound-proof room could not be used. The nerve preparation was placed instead in a double-walled copper box, which was lagged to prevent echo, and stimulation was from a moving-coil loudspeaker mounted opposite a tubular opening in the box. A beat-frequency oscillator (HASKELL and HASKELL, 1955) was used to provide pure tone stimuli in the range 0–20 kc/s. The sound pressure at the tympanal organ was monitored by a Dawe Sound Meter Type 1400 D, which was also used to monitor external noise to ensure this was at all times below threshold level. Waveform and frequency monitoring of the

stimulus was carried out with an oscilloscope. Pressures which just produced a discharge in the tympanic nerve were taken as threshold values, and for each insect three readings at each frequency were taken.

There was a slight difference between male and female responses, the latter being more sensitive, but the difference was not significant when compared with individual variation. The curve in Fig. 1 thus shows the average

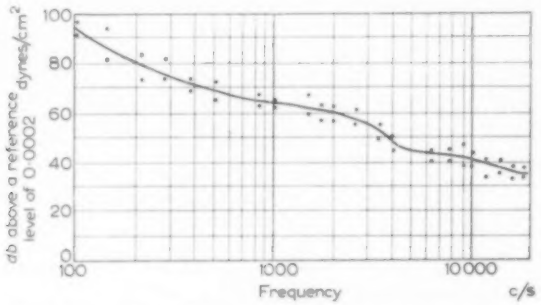

FIG. 1. Graph relating threshold sensitivity in decibels to frequency in cycles per second of a pure tone stimulus in the tympanal organ of *Schistocerca gregaria*.

threshold obtained from three males and three females, together with the scatter at each frequency. This curve shows *Schistocerca* to have a lower threshold throughout the range of frequencies used than *Locusta* as given by PUMPHREY and RAWDON-SMITH (1936a); the difference is greatest at the low-frequency end of the spectrum. This divergence between the present results and those of PUMPHREY is probably due to the different methods of calibrating the stimulus source. On the other hand, lack of a sound-proof room may have caused standing waves and reflections which would tend to augment the sound intensity and it is probable that the true figures, at least for the low frequencies, lie somewhere between the two sets of values. Agreement at the higher frequencies is reasonable, and these are the values used later in this paper.

The experiments were then repeated using a tethered flying locust as a source of stimulation. This insect was tethered to a retort stand by means of a fine thread tied to its pronotum, and a current of warm air directed over it from a small fan and heater. In these conditions, loss of tarsal contact produced sustained flight in the locust, which could then be moved on its support to a given distance from the experimental animal. The wing noise was picked up by a microphone and audio amplifier and fed to the second trace of the oscilloscope, so that direct comparison between stimulus and tympanic action potential was possible.

With these arrangements it was found that action potentials were propagated in the tympanal nerve for every pulse of wing noise emitted by the flying locust, that is, to every complete cycle of wing movement. Fig. 2 shows the correspondence between action potentials (in the lower trace) and flight noise. In quiet conditions, the tympanal organ gave responses of this nature to a locust flying 4 feet away.

This experiment thus confirms those of PUMPHREY as reported in KENNEDY (1951). However, no data is available at present as to the functioning of the tympanal organ of a locust in flight, when conditions would be very different from those in the present experiment.

The preliminary analysis of flight noise was made by using the tethered flying locust and recording the wing noise on a magnetic tape. These recordings were then analysed on the oscilloscope, and Fig. 3 shows the typical waveforms obtained. From the timing wave used it will be seen that the wing-beat frequency was of the order of 17–20 cycles per second, in agreement with the figures of WEIS-FOGH (1956a). A preliminary oscillographic analysis showed that frequencies up to 4 kc/s exist in the flight noise; the waveform of the noise suggested it was made by some sort of impact. The sound pressure of the noise given out by one locust was measured by using the Dawe sound level meter Type 1400 D, and was found to be of the order of 50 dB at a distance of 2 feet on a scale where 0 dB is equivalent to 0·0002 dynes/cm^2.

OBSERVED BEHAVIOUR IN A SWARM AND THE POSSIBLE RELEVANCE OF AUDITORY STIMULI

A. *General Gregarious Behaviour*

The general evidence for this has been discussed in detail by GUNN *et al.* (1948), KENNEDY (1951) and WALOFF and RAINEY (1951). No direct experimental evidence of mutual stimulation by adults has been presented, but KENNEDY (1945) and ELLIS (1951, 1953) have shown that such communication, if it may be so called, exists in locust hoppers. Further examples of gregarious behaviour are given by ZOLOTAREVSKY (1929) and KENNEDY (1951).

In all examples quoted above we may use the term gregariousness in its behavioural sense, "meaning the tendency of swarm members to act in certain ways because other locusts are doing so and not because all are responding to a separate, common source of stimulation" (KENNEDY, 1951). This definition implies communication between individual locusts, as a means of ascertaining what the "other" locust is doing. It has been pointed out that the only sense organs capable on present knowledge of acting as mediators of such communication are the eyes and the tympanal organs; we know that a locust can see another locust in flight and also can hear it at certain distances. Experiments relating to whether locusts can hear their fellows over distances typically found in swarms and whether deafened locusts released in a swarm showed any marked difference in behaviour from intact insects were carried out.

B. *The Edge Effect*

Flying swarms, by virtue of the general cohesion mentioned, show an edge—a boundary—to the swarm beyond which the density of locusts drops off sharply. This boundary is always changing in flight as locusts are driven about by the changes of wind and by the disruptive effects of thermals (RAINEY and WALOFF, 1951). As a consequence of the effectively random orientation within a flying

swarm, individual locusts and groups of locusts constantly approach this edge during flight. However, RAINEY, WALOFF and SAYER (1955) first inferred and later observed that at the edge some reaction occurs which causes the escaping individuals or groups to change course and re-enter the swarm; such a reaction, which they term the "edge effect", would obviously be most important in maintaining the cohesion of the swarm. Later work (WALOFF, 1957; RAINEY, in preparation) has confirmed this.

The "edge effect", as a sequence of behaviour, consists in the escape over the perimeter of a locust or group of locusts, the flight of these away from the swarm, a change in orientation towards the swarm, and subsequent flight on such a path as to enable or cause the insects to rejoin the swarm. Such a sequence of events is capable of being explained in terms of both visual or auditory reactions, or both, provided in each case that the distance travelled by the escaping locusts is not such as to take them far beyond the sight or sound of the swarm. In this connection it was proposed to take measurements of the intensity of flight noise occurring in a swarm, so that some approximate idea of the distance over which an average locust could detect the passage of a flying swarm could be gained.

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C. *Mass-Take-Off Behaviour*

This phenomenon is seen both during the passage of a flying swarm, and also in the morning departure from the roosting site, and has been described in detail by both GUNN (1948) and KENNEDY (1951). On occasion, group take-off is seen to coincide with the passage overhead of a dense stream of flying locusts whose wing-beat noise may act as a stimulus to flight. In some observed cases of mass take-off it would have been impossible for all parts of the group to be affected by any one visual stimulus, for reasons of topography (KENNEDY, 1951), but auditory stimulation might have been possible.

To test these observations, it was necessary to record sounds of flying swarms and play them back to roosting and flying locusts to see if any reaction was produced, and also to use deafened locusts in behaviour experiments.

APPARATUS AND METHODS

To carry out the field research programme derived as shown above, two main instruments were used, a magnetic tape recorder and a sound level meter.

The tape machine was used to make recordings of the sounds of swarms and single locusts in flight, so that an analysis of frequency, waveform and other characteristics could be made later. The sound level meter gave data which allowed the sound energy generated at the source (a flying locust or swarm) to be calculated, which, together with the information on the sensitivity of tympanal organs, enabled the distances over which locusts could hear the flight noise of another locust or a swarm to be calculated.

The tape recorder used was the Vortexion Type 2A Recorder; the amplifier has a flat characteristic up to 10 kc/s, and has a sensitive microphone amplifier, with balanced input, making it suitable for use with the long leads which were required. The microphone used for recording was a Vitavox moving-coil type.

The sound level meter was a copy of the Admiralty Research Laboratory's Sonic Amplifier, and this was matched to a Standard Telephones and Cables omni-directional

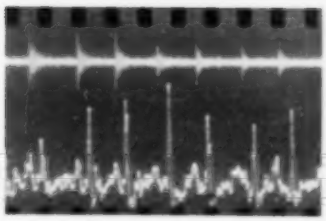

FIG. 2. Oscillogram of flight noise of single flying locust (upper trace) and corresponding action potentials in the tympanic nerve of a nearby Desert Locust.

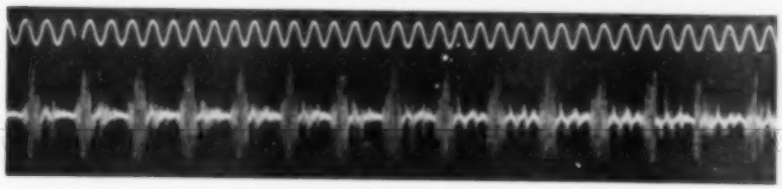

FIG. 3. Oscillogram of flight noise of a single tethered flying Desert Locust. Timing wave 50 c/s.

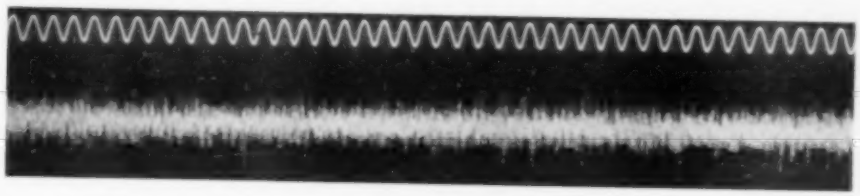

FIG. 4. Oscillogram of random noise produced by flying swarm at a height of 20 feet. Timing wave 50 c/s.

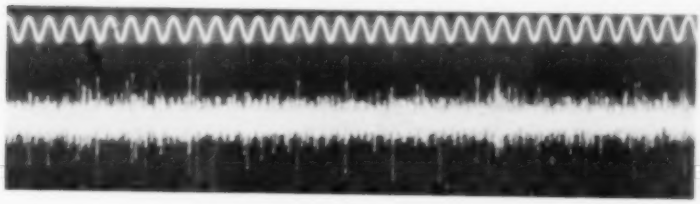

FIG. 5. Oscillogram of general swarm noise with wing-beat noise also present. Timing wave 50 c/s.

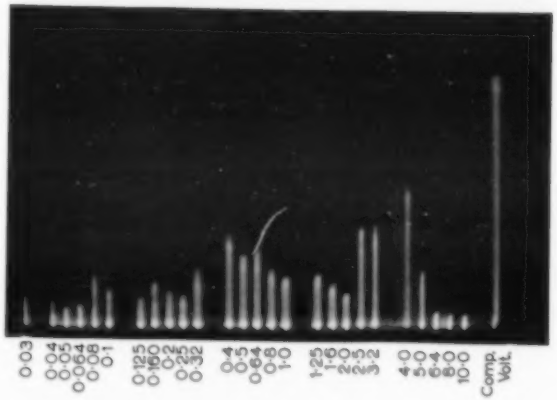

FIG. 6. Audio-spectrogram of general swarm noise.

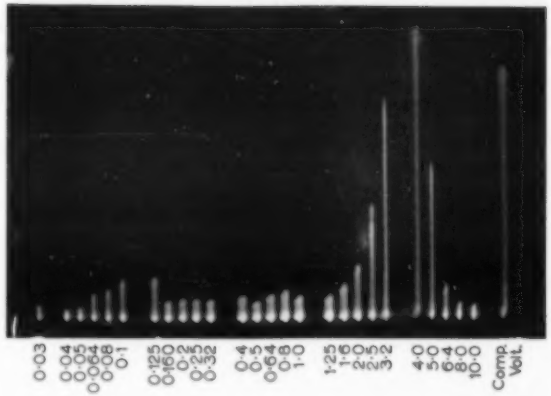

FIG. 8. Audio-spectrogram of wing-beat noise of a single Desert Locust.

In these audio-spectrograms, 26 bands are covered, arranged in 5 groups of 5 spots, with single spots at the extreme left and right. That on the right is for a comparison voltage and in both figures the deflection shown is due to a steady potential of 1·5 volts, used for orienting the photograph. The frequency value for each band is given below it, all values being in kilocycles.

microphone Type 4021G, which is especially designed for tropical use. The whole microphone and amplifier system was calibrated in the anechoic chamber of the Admiralty Research Laboratory at Teddington. Owing to the impossibility of reading the meter accurately under normal fluctuating field conditions, and the fact that the response curve is not flat above 3 kc/s, the figures for sound levels are only accurate to within $\pm 10\%$. At full gain, the amplifier gave a readable deflection of the meter for a sound pressure at the microphone of 0·08 dynes/cm², which is +51 dB on a scale where 0 dB is equivalent to a sound pressure of 0·0002 dynes/cm². All sound levels given in decibels in this paper use this reference level.

From acoustical research (REYNOLDS, 1874; EAGLESON, 1941; and KNUDSEN, 1946) and from work done on the sound fields of stridulating Orthoptera (HASKELL, 1955a) it is known that anomalous propagation of sound near the ground occurs with temperature inversions, and in convective air. In an effort to minimize this effect, a 30-foot portable mast was included in the equipment, with provision for mounting both the recording and sound level microphones on it. This mast enabled these microphones to be positioned above most vegetation, which might otherwise have absorbed and reflected the wanted sounds, and also cleared them from the thermal layers near the ground. As soon as field operations began, it was found that wind produced serious interference with both recording and sound level measurements. The methods used for overcoming this disturbance are described in detail later.

It was found necessary to operate the sound-recording gear at a distance from the ciné and aircraft cameras used to take swarm photographs because of the noise, and it was not practicable at the time to take vertical photographs of the locusts passing over the microphone and no figure for density can be associated with particular noise levels.

RESULTS

A. *Recording and Analysis of Swarm Noises*

Recordings of swarm noises were made with a view to finding if any component present possessed characteristics of intensity and periodicity which would enable it to act as a stimulus to locusts. From our knowledge of the manner in which the tympanal organs of Orthoptera respond to sounds (PUMPHREY and RAWDON-SMITH, 1939; HASKELL, 1956a, b) any stimulus would require to be modulated, or to occur in pulses, to cause a characteristic series of nerve potentials to appear in the tympanic nerve of the receiving locust.

The subjective human impression of a swarm of high density flying overhead at heights of about 30 feet was to most observers that of a gentle swishing noise with no outstanding periodicity. However, low-flying groups or individuals were often heard to produce a distinct clattering noise of a low frequency. These impressions are borne out by analysis of the recordings of the various flight noises. Fig. 4 shows the noise waveform due to a swarm at a height of 20 feet; no repetitive pattern can be recognized, the general appearance of the waveform being that of random noise. However, in certain recordings a component of a repetitive character of frequency between 17–20 cycles per second could be heard above the swarm noise; Fig. 5 shows an oscillograph of such a mixed noise and the repetitive character of this component can be seen. This type of noise was found to be caused by locusts flying with their hind legs trailing; in this position according to WEIS-FOGH (private communication) the wings hit the legs producing a sound,

the frequency of repetition of which is that of the wing beat. When the legs are retracted, the wings no longer collide with them, and it seems probable that during level flight this noise will be absent, and the general noise level lower and of a different character. In the descriptions and discussions on noise and behaviour which follow, the term "wing-beat noise" will be used to indicate the series of noise pulses produced at the wing-beat frequency when the locust is flying with legs down.

Analysis of recordings made during "mass-take-off" flights shows that the wing-beat noise occurs on these occasions; on the other hand, it may well be that during normal level flight no characteristic beat will be present.

TABLE 1—ACOUSTIC POWER OF FLIGHT NOISE OF FLYING SWARMS OF THE DESERT LOCUST

Location of swarm	Date and time of observation	Air Temp.	Rel. Hum.	Wing-beat noise present (+) or absent (−)	Average sound level at microphone in dBs above a reference level 0 dB = 0·0002 dynes/cm²	Estimated acoustic power at swarm corrected for distance and other attenuation, ergs/sec
Mtito Andei, Kenya	Feb. 9th, 1955					
	18.34 hours	21° C	74%	+	56	107
	18.40 hours	21° C	74%	+	54	68
Mtito Andei, Kenya	Feb. 10th, 1955					
	10.21 hours	27° C	60%	−	52	381
	11.07 hours	30° C	50%	−	51	305
	11.10 hours	30·0° C	48%	+	55	339
	11.15 hours	30·0° C	48%	−	52	169
	11.39 hours	30·0° C	48%	+	51	542
Mtito Andei, Kenya	Feb. 12th, 1955					
	11.00 hours	27° C	55%	−	52	678
Mtito Andei, Kenya	Feb. 15th, 1955					
	18.40 hours	22° C	74%	+	53	483
	18.50 hours	22° C	74%	+	55	763
Mtito Andei, Kenya	Feb. 16th, 1955					
	19.30 hours	23° C	75%	−	53	54
Mamba, Kilimanjaro, Tanganyika	Feb. 23rd, 1955					
	09.53 hours	24° C	76%	−	52	381
	10.45 hours	25° C	75%	−	52	169
	11.15 hours	24° C	76%	−	53	54
						Average for all observations 321

The intensity of flight noise when the locust is making the wing-beat noise is much higher than that resulting from level flight. It is therefore to be expected that sound pressure measurements taken under conditions when wing-beat noise is present, for example, during mass take-off, would be higher than those taken when the majority of locusts are in level flight, and this is generally the case as Table 1 shows. It must also be concluded that the data obtained in the preliminary laboratory experiments on the sound pressure of flight noise in single locusts apply mainly to wing-beat noise since flight by tethered locusts (other than in a wind tunnel) is almost always with legs down (KROGH and WEIS-FOGH, 1952).

The sound output of a locust with the legs retracted is very low; on the few occasions in the field when flying locusts with legs up were near enough to both observers and the level meter to provide a checked observation, the noise level was not readable on the meter and was only just audible to human ears at distances of about 6–10 feet.

Audio spectrograms of the swarm noise were made on a G.E.C. 1/3 Octave Band Spectrometer, which has a range of 10 c/s to 100 kc/s. Fig. 6 shows an analysis of the normal swarm noise, with no wing-beat noise present, and is a fairly typical "random noise" spectrum showing components at most frequencies in the bands between 50 c/s to 10 kc/s. Taken in conjunction with the oscillograph of the swarm noise (Fig. 4) these data show that although the tympanal organ of a locust within range would be excited by the noise, no characteristic pattern of impulses would appear in its auditory nerve.

The waveform and spectrogram analysis of flight noise of single locusts is presented below.

B. *Noise Level of Flying Swarms*

Table 1 summarizes the data obtained on the acoustic power produced by flying swarms. In all cases during measurement the sound was monitored, and the occasions on which wing-beat noise could be heard are recorded. Generally its presence is correlated with a high sound energy level.

The variations noticeable in observations taken in the same swarm within a few minutes of each other, when the density of locusts passing over seemed not to have changed markedly, are almost certainly due to variation in attenuation due to atmospheric conditions. In Kenya and Tanganyika, where all observations were made, it is certain that the sound undergoes a great deal of reflection and diffraction by hot air masses and thermal layers and thus suffers attenuation, the amount of which varies not only diurnally with overall changes in temperature and humidity, but from minute to minute as a result of atmospheric turbulence. EAGLESON (1941), in a series of experiments to determine the influence of atmospheric conditions on sound transmission, found signal variations of up to 50% caused by these factors. It is not possible to calculate the attenuation to be expected in the conditions of the present experiments, and large fluctuations in sound level due to atmospheric changes are to be expected. A correction can be made for the attenuation caused by molecular absorption and dispersion in the air. As the air temperatures and

relative humidities for the various observations are known, and a mean frequency character of 4 kc/s for the wing-beat noise can be assumed as a reasonable average value, the molecular absorption can be calculated from the nomogram of KNESER (1933), and the figures given in Table 1 are corrected by this factor. The decrement due to the heat conduction and viscosity of the medium can be calculated by SIVIAN's (1947) treatment of Stokes' classical formula, but the corrections are of the order of 0·001 dB per foot, well within the limits of accuracy of determination.

It was impossible in the present instance to apply the correction factor of GIVENS, NYBORG and SCHILLING (1946) for attenuation due to wind as the intensity/distance data essential to their method could not be obtained. It was also necessary to try to calculate the increment in sound levels caused by wind. This was done by mounting a Sheppard electrical anemometer on the mast at the same height as the recording microphone and taking average sound level readings (*i.e.* background noise of the wind) over periods of 10 seconds, at various wind speeds as indicated by the anemometer. Fig. 7 shows a graph relating sound pressure in decibels to

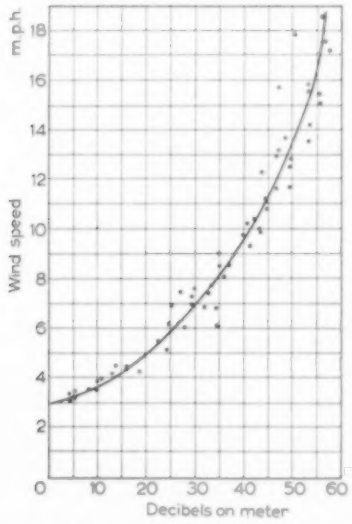

FIG. 7. Graph relating sound pressure in decibels (above a reference level where 0 dB is equivalent to a sound pressure of 0·08 dynes/cm²) to wind speed (at a height of 25 feet) in miles per hour.

wind speed (at a height of 25 feet) in miles per hour. From this it will be seen that the noise due to wind at speeds of 3 m.p.h. or less will not affect the meter, and that providing sound level observations were made with the wind blowing at this speed or less, wind noise could be neglected. This meant that recording could only be done in conditions of calm, and this severely restricted the number of valid readings that could be taken. Wind produced a characteristic noise in the microphone, which was easily recognizable, and this made it possible for the observer who was

monitoring the measurements to reject those in which the effect of wind was apparent.

Fig. 6 gives the frequency spectrum of the swarm noise and calculations based on this show that 60% of the total energy is radiated at frequencies of 3 kc/s and above. This gives an average figure for the acoustic power in this band of approximately 192 ergs per second. This figure relates only to that portion of the swarm near the microphone and it is not possible to correlate this either with swarm density or to form any estimate of the variation in noise output which may occur in the swarm from time to time or from point to point.

C. *Noise Level of Single Locusts*

Table 2 summarizes the data obtained in experiments on the noise output of single, tethered, flying locusts. These experiments were done in tents or in hotel rooms where the necessary low background noise level could be attained; the mean distance from the microphone was taken as the distance of the centre of the flight

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TABLE 2—ACOUSTIC POWER OF FLIGHT NOISE OF SINGLE IMMATURE DESERT LOCUSTS 10 CM FROM THE MICROPHONE

Expt. No.	Sex	Average sound level at microphone in dB above a reference level 0 dB = 0·0002 dynes/cm²	Estimated acoustic power at source, corrected for distance and other attenuation, in ergs per sec
1	Male	64	3
2	,,	71	13
3	,,	68	7
4	,,	62	2
5	,,	70	11
6	Female	70	11
7	,,	62	2
8	,,	70	11
9	,,	67	5
10	,,	68	7
		Average for ♂ = 67 Average for ♀ = 67·4 Average both = 67·2	Average both sexes = 7·2

circle to the diaphragm. These noise levels apply only to the wing-beat noise. There was no significant difference between males and females, although the male average is slightly smaller than the female, which is probably to be expected on account of the differences in size.

Recordings of the flight noise of single tethered locusts were taken for analysis, and Fig. 3 shows an oscillogram of such a recording. The typical repetitive pulse of the wing-beat noise can be clearly seen; the frequency of repetition was found to vary slightly between 17–20 cycles per second. An analysis of the wing-beat noise by the octave band spectrometer is shown in Fig. 8; this is of considerable

interest. Calculations based on this analysis show that approximately 70% of the total energy of the noise is radiated in the band 3·2–5 kc/s and that generally the energy output at all other frequencies is very small. Some recordings, however, when analysed, showed a fairly large component at 80 c/s.

By using the figure of 70% given above, we can now calculate from the average figure of Table 2 the acoustic power due to the flight noise of a single locust over the band 3·2–5 kc/s; this is 5 ergs per second.

D. *Audibility Range of Swarms and Single Locusts*

The data obtained on the frequency/sensitivity relation of the tympanal organ of the Desert Locust, the frequency spectrum of wing-beat and swarm noise, and the acoustic power developed by swarms and by single locusts may now be used to give us approximate measures of the maximum distances over which a single locust can hear other flying locusts or a flying swarm. The actual distances will vary in a complex manner owing to atmospheric conditions prevailing at the time.

The case of single locusts will be considered first; here the average acoustic power in the band 3·2–5 kc/s is 5 ergs per second. Over this band we may take the average threshold sensitivity of the tympanal organ to be 0·063 dynes per sq. cm (Fig. 1). We may now calculate the distance of audibility by using the relationship

$$p = \frac{1}{r}\sqrt{\left(\frac{P.R}{4\pi}\right)}$$

where p = threshold sound pressure of the sound receptor in dynes/cm²,
r = distance of audibility in cm,
P = acoustic power of the source in ergs per second,
R = specific acoustical resistance of the medium at the given temperature.

For flying locusts, the sound waves caused by flight noise may be considered to radiate outwards as spherical waves, and here the denominator of the root fraction remains as 4π. In the case of locusts near the ground or those just taking off from it, the sound waves must be considered as radiating in a hemisphere, when the denominator of the root fraction becomes 2π.

Using the relationship given, the average distance of audibility of one flying locust by another is 0·6 m, or 2 feet approximately. For locusts near the ground the distance becomes 0·9 m (3 feet). This latter value is in general agreement with the figure obtained during the preliminary physiological work. It must be emphasized again that such figures are very approximate and will vary considerably with the atmospheric conditions; this is shown by the variation in the maximum and minimum figures for audibility calculated from the values in Table 2. The maximum distance of audibility by single locusts on present data would be 0·9 m (3 feet) for flying locusts and 1·3 m (4½ feet) for locusts near the ground; the minimum distances would be 0·4 m (1⅓ feet) for flying locusts and 0·6 m (1⅔ feet) for locusts near the ground. These data give an approximate idea of the distances over which locusts can hear certain sounds and thus may be used in discussions of the *rôle* that auditory stimuli play in swarm behaviour.

The estimation of the audibility range of flying swarms is more difficult, as the total sound energy is distributed across a wide range of frequencies. However, 60% of the total energy is given out at frequencies of 3 kc/s and above, and the minimum threshold for this band may still be taken as 0·063 dynes/cm². Using 60% of the average value of the acoustic power of a swarm from Table 1, this gives the average distance over which a flying locust could hear the swarm as 4 m (13 feet), and for swarms near the ground (for example, at take-off) the distance becomes 5·5 m (18 feet). In this case the maximum and minimum distances show an even larger variation; the maximum distances of audibility of a flying swarm by a locust is 6 m (20 feet) or for a locust near the ground 8·5 m (28½ feet), while the minimum distances are 1·6 m (5½ feet) and 2·3 m (7½ feet) for locusts near the ground. It will be remembered that this information relates only to the audibility of dense streams of locusts passing fairly near to the recording microphone. As the density of the recorded streams near the microphone was not assessed, for reasons given above, no relationship between swarm noise and density can be deduced.

E. *Behaviour Experiments*

1. "*Playback*" *experiments*

In these experiments recordings of the wing-beat noise of a single locust were played back to swarms under various conditions.

Experiment 1. Feb. 12th. Roosting swarm 2¼ miles NW. from Mtito Andei, Kenya, towards Makindu, in fairly dense bush. Local time 07.45 hr. Air temp. 23° C. Relative humidity 85%. Cloudy conditions characterized the beginning of the experiment, giving way later to sunshine. No flying at all taking place, and locusts still in trees prior to gliding down to bask. A loudspeaker was placed at the bottom of a tree about 25 feet high which was thickly covered with locusts. The sound played was a recording of the wing-beat frequency of a single locust, and the intensity at the speaker was set to give a calculated sound pressure at the top of the tree of between 53–55 dB. The playback was not commenced until all the locusts flushed during the process of setting up the speaker had re-settled. Ten seconds after the commencement of playback, locusts at the top of the tree began to move restlessly; after 25 seconds some began to take flight, and after 35 seconds locusts were streaming from the tree. Playback was then stopped and gradually in ½–¾ minute flying stopped. The locusts which had taken off only flew short distances and landed again. After an interval of several minutes, during which no flying at all was going on anywhere on the roosting site, the experiment was repeated with the same results. After a further interval, it was repeated again, with the same results. After another lapse of time, during which no flying took place, attempts were made to flush the locusts by banging on strips of wood and by shouting, but no movement was produced. The final two experiments when flying was produced were recorded on ciné film.

Experiment 2. Same swarm as Expt. 1. Time 10.15 hr. Air temp. 27° C, R.H. 60%. Sunshine. By now locusts had descended from trees and were in basking groups on the ground; very little flying was taking place. The loudspeaker was placed near a basking group and the output set to give a calculated intensity at the group of 53–55 dB. On playback no effect at all was observed.

Experiment 3. Same swarm as Expt. 2. Time 10.25 hr. Air temp. 28° C, R.H. 55%. Sunshine. Loudspeaker arranged to give a level of 53–55 dB to group basking on low bush. After 10 seconds all locusts on bush took off.

Experiment 4. Repeat of Expt. 3 to further group on bush. Temperature and humidity the same. No effect was observed. Control experiment to test response to banging wooden slats and shouting produced no effect.

Experiment 5. Feb. 13th. 11.35 hr. Swarm across Nairobi–Mombasa road, ½ mile NW. of Mtito Andei airstrip. Air temp. 31° C, R.H. 40%. Playback of noise of single locust wing-beat noise at highest possible intensity to locusts settled on road produced no effect. That the locusts, which were part of a "drop out" from a flying swarm, were in a fit state to fly was shown by stimulating them visually, either by moving towards them or waving violently, when they at once took off and joined the swarm, which was still passing overhead.

Experiment 6. Feb. 15th. 12.00 hr. Swarm 8 miles NW. of Mtito Andei. Air temp. 30° C, R.H. 44%. Playback experiments of single locust wing-beat noise to group settled on tree 20 feet high produced no effect. This group had only settled on the tree a few minutes when the experiment was started. The swarm was still flying overhead, and there was a steady stream of locusts both settling on, and leaving, the tree.

Experiment 7. Feb. 16th. 07.30 hr. 7 miles SE. of Mtito Andei. Air temp. 23° C, R.H. 78%. Swarm descending from trees in small numbers. Playback of single locust wing-beat noise to dense mass on ground was followed by a thin unsustained take-off after 3 minutes.

Experiment 8. Feb. 21st. 16.30 hr. Swarm at Mamba, on lower slopes of Kilimanjaro. Air temp. 22° C, R.H. 74%. Playback of wing-beat noise to locusts settled on top of conifers about 30 feet high was followed by thin stream-away. There was some scattered flying going on at the time. Powerful audio stimulation produced by a crowd of Chagga natives shouting in unison at the base of the tree produced no effect.

Experiment 9. Feb. 22nd. Same swarm as Expt. 8. 10.37 hr. Air temp. 22° C, R.H. 74%. Playback of wing-beat noise under group of trees about 10–12 feet high produced little effect after 10 seconds. Then a light gust of wind blew up, and, after this had died down, and with playback still proceeding, a large mass take-off occurred.

Experiment 10. Feb. 22nd. Same swarm as Expt. 9. 10.48 hr. Air temp. 22° C, R.H. 74%. The speaker was placed in a tree 8 feet above and 10 feet away from a group basking on the ground; after 10 seconds of playback, a few locusts took off, then rather more, until a thin stream was leaving the group. This ceased when playback was stopped.

Experiment 11. Feb. 22nd. Same swarm as Expt. 10. 11.00 hr. Air temp. 21·9° C, R.H. 73%. Loudspeaker on ground below group roosting in tree 8 feet high. Playback at maximum volume giving a sound pressure in the tree calculated at 65 dB produced no reaction at all.

Experiment 12. March 2nd. Swarm in sisal estate, 7 miles from Korogwe, Tanganyika. 10.20 hr. Air temp. 26·6° C, R.H. 68%. Swarm already flying. Playback of wing-beat noise to locusts settled on sisal produced no reactions.

To summarize these playback experiments: out of 12 observations there was 1 in which a very marked reaction to sound was observed, 2 more in which a small but definite effect was seen, 3 in which an effect was seen but the causes are in some doubt, and 6 in which no effect was seen at all.

2. *Behaviour of deafened locusts*

Experiments were made with deafened locusts, the procedure being to capture a sample of locusts from a swarm in the evening, deafen and mark them, keep them overnight in a cage with adequate food, and release them the following day back into the parent swarm under various conditions. The deafening was carried out by piercing or tearing the tympanal membrane with a small needle; haemolymph exudes from the cut and in a short time forms a clot which surrounds the scolopale organs. This method has the virtue of rendering the organ completely insensitive to sound but at the same time preventing water loss which might occur if the organs were extirpated. Locusts treated in this way seemed to recover almost at

once; they fed normally and no abnormal behaviour was seen. Because of this rapid and apparently complete recovery from operational shock, it was not thought useful to submit the controls to any similar sort of shock, for example, by pricking the abdomen; they were taken from the same sample, and for identification purposes all locusts used were marked with coloured paints on the pronotum.

The locusts, both test and control, were taken from their respective cages and placed in small glass screw-capped vials. The observer released the insects in the required position by knocking the cap off the vials with a stick and allowing the locusts to emerge. The control and test locusts were placed within a few inches of one another either in or as near as possible to a group of basking or resting locusts; these latter could either be on the ground or on bushes, but the former situation was chosen if possible as it afforded a better view. The test insects were watched particularly for any sign of abnormal behaviour while on the ground, for reactions when the basking group took off (either with natural or artificial stimulation), for the manner of flight when airborne, and for reactions, if any, to the flying swarm.

Only those locusts which could be watched through the phases of ground behaviour, take-off and subsequent flight for some distance are included in the observations. Thirty test locusts, 15 of each sex, were thus released and observed; of these, only 4, 2 males and 2 females, showed any abnormal behaviour. Thirty controls were released, 15 of each sex, and none of these showed abnormal behaviour at any stage. Thus only a few insects showed any abnormal behaviour, which seems to indicate that the non-functioning of the tympanal organ does not seriously upset normal behaviour patterns. However, in the four cases in which abnormal behaviour was noted it is interesting that the deficiencies were observed in the normal take-off behaviour.

In two instances, concerning one male and one female, the test and control insects were in a small basking group and had shown no sign of abnormal ground behaviour. After 15 minutes, when the group had not taken off despite several mass take-offs near at hand, they were flushed by the observer approaching them. The group took off at once, and the control insects in each case took off and flew normally with the group to join the swarm. The test insects in both cases stayed on the ground; moreover, as each was closely approached by the observer, they took off, flew 3 or 4 yards and then landed again. This behaviour was not due to their inability to fly but only represented temporary failure to take off, since both insects, on being picked up and flung into the air, flew off quite normally.

A third test insect, a female, joined a basking group, which after some minutes took off normally and flew over the bush where the control still stood; the control flew off with the group, but the test locust had been left behind on the ground and showed no sign of taking-off. It responded normally to visual movements in its range but made no move for half an hour, when it suddenly took off spontaneously, without any apparent external stimulus, flew up normally and joined the swarm.

The last case of abnormal behaviour was in a male; when the basking group to which it belonged was artificially flushed, it made no move to fly, even though the control which was within 9 inches of it on the same leaf flew off. For a further

half an hour it made no response to several flights of locusts near the tree; however, on being flung into the air, it flew quite normally.

TABLE 3—FORCED FLIGHT OF DEAFENED LOCUSTS IN RELATION TO A SWARM

No. and sex	Test (*T*) or control (*C*)	Strength of flight	Reaction to swarm + or −	Remarks
1 Female	*T*	Weak	−	Landed quickly
2 Female	*T*	Normal	−	
3 Male	*T*	Normal	−	
4 Female	*T*	Weak	−	Landed quickly
5 Male	*T*	Normal	+	
6 Male	*T*	Weak	−	Landed quickly
7 Male	*T*	Normal	+	
1, 2, 3 Female	*C*	Normal	+	
4, 5, 6, 7 Male	*C*	Normal	+	

A positive reaction to the swarm means that the locust flew up, joined a group of flyers and orientated itself with them; a negative reaction means that it circled in the group, did not orientate with it, or flew away from it.

TABLE 4—TAKE-OFF OF GROUPS IN RELATION TO STREAMING LOCUSTS NEARBY

Observation	Estimated distance of stream from group in feet	Stream audible (+) or inaudible (−) to locusts on ground	Stream visible (+) or invisible (−) to locusts on ground	Ground locusts roosting (*R*) or settled (*S*)
1	20	−	+	*R*
2	20	−	+	*R*
3	15	+	+	*R*
4	30	−	+	*R*
5	10	+	−	*S*
6	10	+	+, −	*S*
7	10	+	+	*S*
8	15	+	+, −	*S*
9	10	+	−	*S*
10	25	−	+	*S*

Note: (*a*) In all cases observed here, the group on the ground took off.
(*b*) In certain cases, a proportion of the locusts on the ground could see the stream overhead and others could not; in these cases the sign +— is used.
(*c*) Roosting locusts are those which arrived on the site the previous day. Settled locusts are those which have temporarily dropped out of a flying swarm.

A small experiment was done on releasing deafened locusts into a flying swarm. The locusts used could not be released in the swarm from which they were captured, and they had to be kept for 3 days in a deafened condition before being used; however, their behaviour in the cage prior to release appeared quite normal.

Observers were on a road just below the crest of a hill, over which the swarm was passing; thus observation of the locusts for some distance was possible. The procedure was for the releaser to shout out the type (either test or control) and sex of the locust, then fling it into the air against the wind and thus against the direction of the locust streams overhead, which were at heights varying from 15–30 feet above the road. Observations of the type and direction of subsequent flight were made and recorded as long as the test locust could be seen. Table 3 summarizes the results of this experiment.

3. *Take-off of groups of locusts in relation to other locusts streaming nearby*

Take-off of basking or settled groups in relation to dense streaming of locusts overhead has been reported by GUNN (1948) and KENNEDY (1951). Table 4 gives some relevant data on this phenomenon which was observed in detail ten times.

Finally, reference must be made to observations of the "edge effect". In cases where the effect was seen convincingly by the author the distances which the escaping locusts flew away from the swarm before they turned back varied from 2 to 25 yards.

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DISCUSSION

The methods available in the investigation of flight noise only allow us to assess in a rather approximate manner the sound pressure levels present at a particular point in the particular swarm to which they apply; this is, however, sufficient for us to make some general deductions as to the probable range of auditory reactions in a swarm, which have been done above. These figures can be used to discuss whether audible stimulation from the flight noise can play a part in some of the major behaviour patterns associated with flying swarms—general cohesion, edge effect and mass take-off. In this connection we shall also consider the significance of the two types of noise output caused by flying locusts—the "general swarm noise" and the "wing-beat noise".

General cohesion

As far as general cohesion is concerned, in the absence of information about the effect of the various sensory inputs to a locust on its flight, it must be assumed that any method by means of which a locust can be aware of its companions may be useful in aiding coherence. In considering reception of auditory stimuli by locusts through the tympanal organs we must bear in mind the nature of the two types of stimuli to which they are subjected; these are the general swarm noise, and the wing-beat noise. The former, although it will excite the tympanum, produces only asynchronous responses which will vary in frequency in an irregular manner; the latter will produce responses synchronous with the frequency of the wing-beat.

Our present knowledge of the effect of the form of the auditory stimulus on the behaviour pattern released by it is small as far as invertebrates are concerned; BUSNEL and LOHER (1954) have shown that certain male Acridids can be attracted by artificial sounds which only bear a slight resemblance to the natural sound, the

stridulation, which normally provokes the response. HASKELL (1956a, b) has shown that certain complex behaviour patterns are released in females of various species of Acridid by artificial sounds, provided that the pulse repetition rate of the artificial stimulus is similar to that of the natural one. Evidence from the present work and from WILLIAMS (1933) indicates that random noises such as banging and shouting do not produce flight response in the Desert Locust, although it is certain that they do excite the tympanal organs. It is thus likely that the general swarm noise will not elicit any specific reactions from a locust hearing it, but this is not to say that it will not contribute to the maintenance of central excitation. On the other hand, the wing-beat noise has characteristics by virtue of which it could act as a stimulus for a specific behaviour reaction. In the light of this inference we can now consider the noises likely to act as stimuli to locusts in a swarm.

Figures for the volume densities of swarms of the Desert Locust have been given by GUNN (1948), RAINEY and SAYER (1953), and RAINEY (1955), and range from 14 locusts per cubic metre to 0·01 locust per cubic metre. The latest evidence suggests, however, that densities of the order of 0·1 locust per cubic metre are representative of the denser parts of flying swarms. For the purposes of discussion we will consider two densities, the first of 1 locust per cubic metre, the second of 0·1 locust per cubic metre. In the first case the average distance of a flying locust from its fellows will be 1 metre (3·3 feet) and in the second 2·2 metres (7·3 feet). According to the figures for audibility range of the two types of flight noise given above, in swarms of the first density a locust would hear the general swarm noise and probably the noise of its nearest neighbours if these were making the wing-beat noise, but in swarms of the second density a locust would only hear the general swarm noise. Since densities of this second order are now thought to be representative even of dense swarms (RAINEY, 1955), it is probable that during normal flight of a swarm, locusts in it will only be subject to the auditory stimulus of the general swarm noise; during the bunching-up which occurs at take-off or landing, however, they are liable to hear wing-beat noise from their nearest neighbours.

Recent work by WEIS-FOGH (1956a, b) indicates that sound stimuli have little effect on the maintenance of flight in the Desert Locust; sudden high-pitched noises intensify flight, but unless the insect is decerebrate the effect is short lived. There was no response from test insects to the noise of another locust's wing-beats. These experiments were directed mainly towards *changes* in the flight performance, whereas, as WEIS-FOGH points out, a characteristic of the migration flights of the Desert Locust is the *constancy* of flight performance in the insects, and he goes on ". . . flight itself, once brought about, tends to reduce the level of environmental stimuli necessary for its continuation". It seems possible that the maintenance of such steady state flight might be correlated with a continuous stimulus such as the general swarm noise.

WEIS-FOGH's work was mainly directed towards investigation of the systems of initiation and maintenance of flight, and little is known about the behaviour during

flight or the mechanisms that might control it. In many Orthoptera, the tympanal organs play such an important rôle in the initiation and orientation of locomotor movements that the possibility that they influence behaviour in flight cannot be lightly dismissed. Unfortunately the necessary behaviour work is extremely difficult, and the few results in this direction given above (see Table 3) do no more than hint that further experiment may be rewarding.

It seems unlikely that the promotion of general cohesion in a swarm can be mediated by one sensory modality alone, and common sense insists that visual as well as acoustic stimuli must play an important part in the process. The elucidation of the part played by swarm and wing-beat noise in swarm cohesion must thus await the investigation of the integration of peripheral stimuli in the locust.

Edge effect

The "edge effect" as a sequence of behaviour has been described above. In observations of definite cases of this phenomenon the distances which individuals or bands of locusts flew away from the edge before turning varied between 2 and 25 yards. These distances are mostly larger than those over which a locust could be expected to hear the noise of the swarm it had just left.

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A single tympanal organ is capable of a directional response because it is a "displacement receiver" (PUMPHREY, 1940) and displacement is a vectorial quality of sound. It may be argued that a locust leaving the edge of the swarm would thus be directionally stimulated by the general swarm noise, which may engender behaviour causing it to return to the swarm. This possibility cannot be completely discounted until more work is done, but one difficulty is that a swarm presents, not a point source of sound, but a source very large indeed when compared to the receptor. Thus for a locust 10 yards from the edge of a swarm only 440 yards in length—a very small swarm indeed—the angle subtended by the source at one tympanal organ is 87° approximately. From PUMPHREY's graph of variation of sensitivity with direction, such an angular dispersal of the source would make it virtually impossible for the insect to orientate to it. Furthermore, the "edge" of a swarm is not a clearly demarcated boundary but is represented by an area in which the density of locusts falls off sharply. A single locust traversing this area might thus fly quite close to a number of other locusts whose sound output would tend to confuse reorientation to the swarm as a whole.

The great variation in the distances which locusts were observed to fly away from the swarm before turning is an added complication. It is not possible to deny, in the absence of any knowledge of the conditions of sound attenuation in each particular instance, that the turning point of each flight may have marked the point at which the locust ceased to hear the swarm flight noise, and the cessation of this stimulus may initiate the behaviour necessary for returning to the swarm.

Further speculation on this behaviour is precluded by the lack of knowledge of the flight behaviour (as distinct from the flight performance) of locusts in response to various sensory stimuli.

Mass-take-off behaviour

It is in the repeated observations of mass-take-off behaviour that most direct evidence of gregariousness has had its genesis. Strong subjective impressions of gregarious behaviour at this time have been formed by almost all observers, and it is therefore disappointing that the experimental evidence on the rôle played by auditory stimulation from flight noise, gained in the present work, is somewhat contradictory.

There are two aspects of take-off behaviour which differ at least in their magnitude. One, mass take-off, characteristically affects large sections of a roosting swarm, and leads to rapid evacuation of the site. The other, group take-off, affects small groups of locusts, and appears amongst those insects settled from a moving swarm when they take-off to rejoin that swarm. The experiments on take-off behaviour described above thus include both these types. However, there is as yet no reason to suppose that the sensory stimuli leading to take-off in these two types of behaviour differ in any way; it seems more reasonable to suppose that the difference lies in the reactivity of the locusts, which may be affected by many factors, external, such as temperature, or internal, such as metabolic causes. The discussion is based on this probability.

From the playback and control experiments carried out there can be no doubt that random noise such as shouting and banging wooden slats had no effect on locusts at all; this confirms WILLIAMS's (1933) experience and experiments. It is, of course, also in line with what we know of the physiology of the insect tympanal organ; the only type of sounds which will produce characteristic discharges in the auditory nerve are those in which the sound is modulated or occurs in regular pulses. This may explain the success of one of the experiments of WILLIAMS, in which he caused large numbers of locusts to take flight by drawing a file over the teeth of a saw; here the impact of each tooth as hit by the file would produce a pulse of sound, and if the frequency of the pulse repetition was of significance to the locusts, they may have taken flight due to this. The only sound known to be made by the Desert Locust with any characteristic pulse rate is the wing-beat noise. Most locusts taking off appear to make this noise until they have gained a certain height and thus it would be reasonable to look for effects of stimulation from this noise during take-off.

The first experiment on playback of the noise to roosting locusts produced an immediate and remarkable response, and the circumstances of the test left no doubt in the minds of the observers that the sound was responsible for the effects noted. There is, however, a measure of contradiction in the results. If wing-beat noise stimulates take-off, why did the initiation of flight by playback not produce complete evacuation of the tree, since the first locusts to be flushed would presumably themselves produce the noise, thus inducing more to take off, a process which would be expected to clear the tree?

It is possible to give a reply to this criticism; the experiment was done on locusts which had not yet descended from the tree tops to bask, and whose body temperature would thus be expected to be below that required for active flight

(GUNN, 1948), particularly as at the commencement of the experiment meteorological conditions were cloudy. As recorded in the observations, the locusts induced to take off only flew short distances and then landed again, and the general course of this flight was down towards the ground, as is to be expected for the time of day. In this case it is doubtful whether much wing-beat noise was produced as most of the descending locusts glided down. It is also to be expected that most of the locusts which did take off were those which were physiologically more active than their colleagues, and so a limited response was to be expected. In fact, the slow dying down of the flight response noted when playback was stopped is exactly the type of behaviour to be expected under these circumstances. A similar explanation may be advanced to explain the results of experiment 7, where playback to a dense mass on the ground produced a thin unsustained take-off. The air temperature was 23° C, and this on GUNN's figures (1948) is probably only just sufficient for stream-away or constant flight; hence we may suppose that the response obtained was due to those locusts completely "ready" for flight, and the lack of response of the majority was attributable to their lack of readiness. In experiment 10 another thin stream was produced by playback which ceased when the stimulus was stopped; here again the air temperature was low, 22° C, and probably only a proportion of the group was ready for take-off. This explanation may be criticized on the ground that it is possible for swarms to leave the roosting site when the air temperature is only 17–18° C, provided the sun is shining so that the locusts' body temperature is kept up by absorption of radiant heat (GUNN *et al.*, 1948). But in these cases the locusts *behave* as if the air temperature was high; in the present experiments the behaviour of the locusts was that usually observed when body temperature is low, that is, gliding down from trees or basking on the ground.

On consideration of the remainder of these playback experiments, one is at once struck by the very variable nature of the results. For example, experiment 2 was performed in the same swarm and under the same weather conditions as experiment 1; the main difference in environment was that at the time of the second experiment the air temperature had increased from 23° C to 27° C, which should have made more locusts ready for take-off, but no response to playback was observed. However, playback 10 minutes later to another group of the same swarm, with the air temperature now 28° C, was followed by take-off. Such results indicate that the variation is to be looked for in the locusts themselves, and suggests that a combination of stimuli is necessary for take-off. In some of the experiments the operation of a second factor is clearly seen; for example, in experiment 9 playback had no effect for 10 seconds, then a light gust of wind blew up and when this died down and with playback still proceeding, a large mass take-off occurred. KENNEDY (1951) mentions that "a sudden lull after a period of strong wind led to mass taking off by settled locusts" and goes on to postulate that "the 'damming-up' of spontaneous flying by wind leads to a situation in which a slight lull . . . releases flying with unusual unanimity". In the present case the swarm was on the side of Mount Kilimanjaro and the wind had been light and variable all day, and take-off due to changes of wind had not been noticed. But when playback was going on,

a change in wind velocity occurred and was followed by mass take-off; this suggests that a combination of external factors affects take-off.

That one of these factors is vision there can be little doubt; it was always found possible in the field to flush a group of locusts by causing a movement in their field of vision. The work of BURTT and CATTON (1954) has shown that the visual acuity of the compound eyes of the locust is of the order of 0·16° of arc, and certainly very small movements produced immediate flight responses in most cases. However, in groups of locusts which can be afterwards shown to be capable of take-off and flight, continued take-off of individuals throughout the group does not initiate mass take-off. The work of WULFF *et al.* (1955) suggests that one possible explanation of this is a quantitative deficit in the visual stimulus; however, it is more likely that the reason lies in the temporary unreactivity of locusts to stimulation in one sensory mode, which will call for a combination of various stimuli to effect take-off.

The interaction of audio and visual factors in take-off seems to be underlined by the results of the experiments on deafened locusts. In general, deafening produced no marked abnormality of behaviour and thus acoustic stimuli cannot be put down as a prime factor in take-off patterns; but of the deafened locusts which did show abnormal behaviour, all experienced difficulty at the take-off phase, although all were capable of normal flight when flung into the air. In some cases, however, deafened locusts in basking groups took off normally with the group, presumably in response to mutual visual stimulation.

This variability in the combination of stimuli necessary for take-off is also seen in Table 4, which shows the take-off of roosting or settled groups in relation to nearby streaming away of locusts. Although a combination of audio and visual stimuli always produced take-off in the groups concerned (Observations 3, 6, 7, 8), lack of either one or the other (Observations 1, 2, 4, 5, 9, 10) did not prevent it.

It seems probable that the metabolic state of the locusts at the time of take-off would be a third important factor which must be considered; this factor may particularly apply to examples of temporary individualistic behaviour when single locusts seemed to act quite independently of the group in which they found themselves. The fact that such locusts often needed tactile stimuli to produce take-off seems to indicate that the threshold sensitivity of the "take-off factor" was in this case very high, and this fact may be correlated with changes in metabolism produced by flight. There is some evidence (WEIS-FOGH, 1950) that the readiness of locusts to fly may be related to the duration of previous flight. It is remarkable, however, that such individualistic locusts were rarely left behind by the swarm, an indication that the effect may only have been temporary.

Finally, reference must be made to the apparent localization of take-off stimulus to a group; this phenomenon is complicated by the variable size of the group, which may be a few locusts gathered on one branch of a tree, or a large basking group containing several thousand locusts. The apparent simultaneous take-off by such a group, without causing the least disturbance to other nearby groups, is a well-known feature of the swarm behaviour, and has been noted by GUNN (1948),

KENNEDY (1951), and others. However, if it be postulated that one of the initiating factors in take-off is an acoustic stimulus in the form of the wing-beat noise, this variability in the size of the group taking off may correspond with the range of the stimulus, which would vary according to the atmospheric conditions in the locality of the group. On the evidence given above, the range of the wing-beat noise for locusts on or near the ground can vary from 1 to 5 feet approximately. The difficulty in this explanation is that one would expect the effect to grow with the numbers taking off, and thus to spread the stimulus wider and wider, which evidently does not occur; this limitation can only be explained if some factor such as the metabolic "readiness for flight" of nearby locusts is invoked.

Thus, to sum up on the question of acoustic stimuli and mass take-off, it may be said that evidence exists which shows that, in certain circumstances, wing-beat noise produced by locusts can act as a stimulus to take-off by other locusts, but that its effectiveness appears to depend upon a combination of factors, some of which may concern other sensory inputs to the locusts and some their metabolic state.

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SUMMARY

1. The response of the tympanal organs of *Schistocerca gregaria* (Forskål) has been investigated by electrophysiological methods, and the relation between threshold sensitivity in dynes/cm² and the frequency of a pure tone stimulus in the range 100 c/s–20kc/s has been determined.

2. The response of the tympanal organs of *Schistocerca* to the flight noise of another locust has been determined, and has been shown to consist of bursts of nerve impulses at the frequency of the wing cycle of the stimulating locust.

3. Recordings and analyses were made of the sounds of swarms and single locusts. From these it was shown that the sound output due to flight noise in a single locust changes in character and intensity according to the position of the locust in the air. Locusts with hind legs trailing emit a sound which has an average acoustic power at the source of 7·2 ergs per sec, and has a pulse repetition rate equal to the wing-beat frequency, about 17–20 cycles per second; 70% of the total energy of this sound is radiated in the band 3·2–5 kc/s. This sound is named the "wing-beat" noise. Single locusts with legs retracted in the normal flight position produced a noise of such low intensity that it could not be measured with the apparatus available.

Swarms of locusts produced a noise, the "general swarm noise", which has a spectrum similar to random noise, and an average acoustic power at the source of approximately 321 ergs per sec in the particular cases observed.

4. The approximate distances over which single locusts can hear flying swarms and flying single locusts, when these are (*a*) in the air, and (*b*) near the ground, were calculated from the foregoing data. The effect of atmospheric conditions on the variability of these distances is discussed.

5. Behaviour experiments, where recordings of "wing-beat" noise were played back to swarms under various conditions, are described as well as experiments with deafened locusts under normal and forced flight conditions. Also observations

relevant to swarm behaviour and auditory stimulation by flight noise are recorded. It was found that playback of "wing-beat" noise stimulated take-off in settled or roosting locusts under certain undefined conditions, and that deafened locusts, although able to fly normally when thrown into the air, occasionally showed failure to take-off. Deafened locusts when flung up into a swarm reacted normally to it in the majority of cases.

6. The data obtained as outlined above are discussed in relation to three major behaviour patterns observed in swarms: general cohesion, the "edge effect", and take-off behaviour.

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GROWTH-RETARDING EFFECT OF CARBON-DIOXIDE ANAESTHESIA ON THE GERMAN COCKROACH*

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Abstract—German cockroach nymphs exposed to a high concentration of carbon dioxide for 3 minutes weekly until maturity were retarded in growth by 14% to 53% compared to nonanaesthetized controls, as determined by the time required to moult to adults. There was no apparent correlation between diet and the carbon-dioxide effect. The adult weight of roaches subjected to weekly anaesthesia during the nymphal period was less than that of nonanaesthetized roaches. Nymphs reared as isolated individuals were retarded by carbon dioxide relatively as much as nymphs reared in groups. Nymphs kept under constant carbon-dioxide anaesthesia for 2½ hours became greatly distended by gas, remained paralysed, and died within several days. The retardation and lethal factors seem to be carbon-dioxide specific rather than a general immobilization, nervous, or oxygen-deficit effect inasmuch as nitrogen was relatively innocuous. Consideration was given to the possibility of the formation of a toxic carbon-dioxide compound which is retained by the blood.

A GENERAL practice in entomology laboratories is the use of tank carbon-dioxide gas to immobilize insects. To cite only a few examples, the gas is used to anaesthetize the animals during surgery (WILLIAMS, 1946); to prevent their escape while being weighed (HILCHEY, 1953); to facilitate the removal of egg capsules (ROTH and WILLIS, 1955); to ensure uniform application of test insecticides (TATTERSFIELD and KERRIDGE, 1953); or to stimulate oviposition following artificial insemination (MACKENSEN, 1947).

The stimulatory effects of carbon dioxide on spiracular opening and respiratory movements, and its attractant qualities, are quite well known (see ROEDER, 1953). Although for some insects the toxic side-effects of ether narcotization are recognized (CORNMAN, 1944; MACKENSEN, 1947), carbon dioxide when applied for short periods of time is generally thought to be innocuous. SCHREUDER and DE WILDE (1952), in commenting on the remarkable qualities of carbon dioxide, state that it is a powerful, completely reversible anaesthetic which can be applied for hours or even days.

However, WILLIS (1925) studied recovery times for certain grasshoppers following exposures for different periods to various carbon-dioxide tensions, and recorded that an exposure of 5 hours or more resulted in death. BEADLE and BEADLE (1949) reported that there are interspecific differences for immobilization

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time and recovery among various insect larvae and crustacea. In our laboratory, during the course of infection experiments on 15 species of cockroaches with Laboulbeniales, Dr. A. G. RICHARDS noted that certain species (not generally kept in laboratories) failed to recover from short-period anaesthesia. Dr. A. F. O'FARRELL (personal communication) has observed that carbon-dioxide anaesthesia results in great variability of an erratic and unpredictable kind in the moulting cycle of *Blattella germanica*.

About one-third of the wild strains of *Drosophila* caught in France and several other countries are peculiarly susceptible to carbon dioxide, the sensitive individuals failing to recover from an exposure which is completely reversible for resistant flies (L'HÉRITIER and TEISSIER, 1937; KALMUS, KERRIDGE, and TATTERSFIELD, 1954). The factor responsible for the sensitivity is a virus which in nature is inherited *via* the eggs and sperm but which in the laboratory can be transmitted to resistant flies by injecting suspensions of susceptible-fly tissue (SIGOT, 1954). The lethal factor is usually expressed in a discrete all-or-none fashion, although sensitive flies can become adapted to resistance by progressive exposures (TATTERSFIELD and KERRIDGE, 1955).

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There are a few known cases in which short exposure to carbon dioxide is apparently reversible but is followed by delayed side-effects. Thus, JANISCH (1924) exposed females of *Ephestia kühniella* to the gas and found that they did not mate or oviposit. He interpreted this as "artificial ageing". FYG (1950) found that in queenless worker bees of unknown ages, after exposures of up to 90 seconds, the ovaries and fat bodies remained underdeveloped, the pharyngeal glands inactive, and the bees did not perform their normal duties of drawing comb foundation, storing sugar, or building queen cells. RIBBANDS (1950) agreed that elimination of brood-rearing and wax-secretion was tantamount to ageing, but he felt that the reduction of life expectancy shown by his carbon-dioxide anaesthetized bees could be attributed to an induced change in behaviour. Anaesthesia did not give rise to abnormal behaviour but simply changed the behaviour from one normal pattern to another. MACKENSEN (1947) found that carbon-dioxide anaesthesia of artificially inseminated queens reduced the average age at initial oviposition nearly to that of naturally mated queens, and it also increased the number of laying queens. The carbon dioxide which accumulates during pupation of silkworms under crowded conditions is said to result in the spinning of cocoons of inferior quality (YAMAFUJI, YONEZAWA, and HIRAWA, 1935).

Studying the susceptibility of different species of insects to a series of poisons, BUSVINE (1942) reported that there seems to be one order of susceptibility for fat-solvent vapours and a different order for gaseous fumigants. He found that the order of resistance to carbon dioxide is the same as that to the fat-solvent vapours, the median lethal exposure to carbon dioxide being 15, 30, 54, 68, and 213 hours for *Cimex lectularius*, *Tribolium castaneum*, *Ephestia kühniella*, *Calandra oryzae*, and *Calandra granaria*, respectively. The ability to tolerate carbon dioxide was found to be a limiting factor in the distribution of pests of stored wheat by HOWE (1943).

Attempts to analyse the physiological mechanism of carbon-dioxide anaesthesia have led to conflicting views. Thus, it is well known that high carbon-dioxide tensions can induce opening of the spiracles in various insects, but it is clear how important is the stimulus of ordinary carbon-dioxide tensions for normal spiracular action. The point is at least somewhat complicated because SCHREUDER and DE WILDE (1952) reported that the control of respiratory movements by thoracic ganglia is affected by carbon dioxide in a different manner from the control by abdominal ganglia.

It has also been suggested that lasting effects of carbon dioxide may be no more than tissue injury resulting from accumulated acid metabolites following anoxia. Although cogent evidence for this view is lacking, RIBBANDS (1950) reported that carbon dioxide and nitrogen had similar effects on the behaviour of honeybees; SIMPSON (1954) reported that carbon dioxide, nitrous oxide, and ammonium nitrate fumes had similar effects on bee behaviour; and BAILEY (1955) concluded that the death of *Calandra granaria* in closed vessels was due to oxygen depletion rather than to carbon-dioxide excess.

The foregoing review of the literature indicates that aside from the carbon-dioxide lethal factor (virus) of *Drosophila*, and the artificial ageing (change in behaviour pattern) of honeybees and certain grain pests, short exposures to carbon dioxide have been generally practised for anaesthetic purposes on the assumption that there are no harmful after-effects.

The present study was undertaken to determine the effect of short-exposure carbon-dioxide anaesthesia on the growth of the German cockroach because it was observed that (*a*) nymphs failed to recover from anaesthesia of 2 hours' duration, and (*b*) nymphs that were anaesthetized once a week for transfer to weighing vials failed to grow as rapidly as their unweighed controls.

MATERIALS AND METHODS

All experiments were done with the German cockroach, *Blattella germanica* L. The gases, carbon dioxide, nitrogen, and 95% nitrogen–5% carbon dioxide, were obtained from commercial sources in high-pressure cylinders. Both experimental and control insects were reared in groups of varied numbers in brass gauze-topped, glass, pint jars in a constant temperature cabinet at $27{\cdot}5 \pm 0{\cdot}5^{\circ}$ C except in one instance explained below. The newly hatched nymphs from several egg capsules were pooled and apportioned at random to the various pint jars. During the early experiments, the CO_2 was blown rapidly from the cylinder into the jars through the screen tops for 5 seconds and allowed to remain there for 2 minutes. Then compressed air was blown in for 5 seconds. The roaches revived during the next 3 minutes. In the later experiments CO_2 was blown in through a flowmeter, 3 litres during a period of 10 seconds, and after 2 minutes was blown out with compressed air for 5 seconds. The results of the two methods were comparable. Thus the time of exposure to concentrated CO_2 was not over 3 minutes weekly. Weekly anaesthesia was continued until the last insect in each jar had matured. Since it was extremely inconvenient to weigh the controls that were not anaesthetized,

the number of days required by each insect to reach maturity was taken as the end-point. The final figures were calculated as averages of these figures.

Several diets were used: a crude diet of ground dog biscuit ("Morton's Kibbies"); a semi-synthetic diet containing casein, linoleic acid, crystalline B-vitamins, etc.; a completely synthetic diet containing amino acids, linoleic acid, crystalline B-vitamins, etc. (formulae by courtesy of Dr. H. T. GORDON); and both the semi-synthetic and synthetic diets with different levels of either corn oil ("Mazola") or linoleic acid (Nutritional Biochemicals Company).

Altogether there were 57 groups with a total of 692 cockroaches.

RESULTS

A 3-minute exposure to concentrated CO_2 gas once each week consistently prolonged the average time taken to reach maturity by amounts ranging from 14% to 53%. The absolute number of additional days required by anaesthetized roaches ranged from 6 to 25. The basal time for nonanaesthetized roaches ranged from 41 to 90 days, depending on the diet.

The delay following anaesthesia with 95% N_2–5% CO_2 was considerably less, ranging from 4·5% to 8% in three groups, but there was an acceleration of 4% in one group. One group anaesthetized with "pure" N_2 required the same average time as the nonanaesthetized controls.

The CO_2 effect could not be correlated with the diets that were used.

An additional factor which became apparent was that CO_2 treatment was usually accompanied by a higher survival rate. Out of 14 paired groups, the survival ratio of the anaesthetized ones until maturity was higher in 9 groups, equal in 3, and lower in 2, compared to the control roaches. The reason for this superior survival is not known, but direct observation as well as results from rearing in isolation both show that it is not due to cannibalism.

Table 1 (p. 80) summarizes the data obtained on all roaches reared in groups of 13 to 20 with the diet as the variable factor when the gas was blown into the jar for 5 seconds and replaced with air after 2 minutes.

When corn oil was substituted for linoleic acid (the product used was 60% linoleic acid), all else being equal, growth was improved. In consideration of this, and of BUSVINE's (1942) report that the order of resistance to asphyxiation by CO_2 is the same as to fat-solvent vapours, it seemed worthwhile to vary the lipid composition of the diet. Casein diets were made containing 1, 5, 10, or 20% corn oil or 1, 5, 10, or 20% linoleic acid, the lipid being added at the expense of the non-nutritive filler so that the other components remained constant. Three litres of CO_2 were blown into the jars in 10 seconds and replaced with air after 2 minutes. The results are summarized in Table 2 (p. 81).

Increasing the lipid content slowed down growth, linoleic acid being more effective than corn oil, but there was no consistent trend in the susceptibility to CO_2. On 20% linoleic acid, growth was greatly retarded, and a casual inspection of the figures for percentage delay suggests that this diet protected against the CO_2 effect. But this is merely a reflection of the fact that the absolute delay, which was

essentially the same as on the other diets, is a smaller percentage of the long growth period enforced by that diet.

The roaches on these fat-variable diets were weighed after they matured. With one exception (diet C-l-10), the weight of the anaesthetized roaches always averaged less than that of the controls. In Table 2 the number of females is expressed as a

TABLE 1—COMPARISON OF DAYS REQUIRED TO MATURE AFTER THREE-MINUTE ANAESTHESIA WEEKLY

Group number	Diet*	Anaesthesia	Days to mature		Additional time		Survival ratio†
			Range	Average	Absolute	Relative	
(1)	DB	—	40–50	46	—	—	12/16
(2)	DB	CO_2 (8 times)	44–63	52	6	14%	14/16
(3)	C-l	—	39–47	42	—	—	18/20
(4)	C-l	CO_2 (9 times)	50–75	59	17	41%	20/20
(5)	C-l	N_2+CO_2 (7 times)	39–51	44	2	5%	18/20
(6)	S-l	—	54–84	67	—	—	12/20
(7)	S-l	CO_2 (11 times)	74–97	85	18	27%	15/20
(8)	S-l	N_2+CO_2 (10 times)	52–88	70	3	5%	16/20
(9)	S-c	—	51–77	62	—	—	10/20
(10)	S-c	CO_2 (10 times)	66–97	79	17	27%	18/20
(11)	S-c	N_2+CO_2 (9 times)	59–84	67	5	8%	10/18
(12)	C-l	—	34–58	47	—	—	15/18
(13)	C-l	CO_2 (8 times)	58–89	72	25	53%	15/18
(14)	C-l	N_2+CO_2 (8 times)	34–55	45	(−) 2	(−) 4%	16/18
(15)	C-l	N_2 (8 times)	42–58	47	0	0	17/18

* DB = dog biscuit; C-l = casein diet containing 1% linoleic acid; S-l = amino acid diet containing 1% linoleic acid; S-c = amino acid diet containing 1% corn oil.

† The numerator is the number of insects which lived to maturity while the denominator is the number of nymphs at start of experiment.

fraction, the numerator being the number with formed oöthecae and the denominator being the total number of females. It is obvious that the lighter weight of the females is not a result of the lack of extruded oöthecae ; however, no effort was made to determine if the size or number of eggs was reduced or if the body-weight exclusive of ovaries was reduced.

The possibility that CO_2 affects the social behaviour of the cockroaches was considered. CHAUVIN (1946) found that isolated roaches grow more slowly than grouped roaches, but that adding excreta to the food improved the growth of the isolated insects. The possibility of a trophallaxis was suggested by ROTH and STAHL (1956), who noted that the tergal and cercal secretions of roaches accumulate on isolated individuals but may be rubbed or eaten off by grouped insects.

In a preliminary experiment in this laboratory, single nymphs were put into small cages and reared at room temperature (about 23–26° C). In this instance there was some overlapping, but the average time taken by anaesthetized roaches was 19% longer (15 days) than that taken by the control roaches.

TABLE 2—COMPARISON OF DAYS REQUIRED TO MATURE AFTER THREE-MINUTE ANAESTHESIA WEEKLY ON DIETS OF INCREASING FAT CONTENT

Group number	Diet*	Anaesthesia	Days to mature		Additional time		Survival† ratio	Numbers and average weights			
			Range	Average	Absolute	Relative		Males	mg	Females‡	mg
(16)	DB	—	37–57	43	—	—	13/15	6	50	7/7	112
(17)	DB	CO_2 (9 times)	46–64	52	9	21%	12/13	6	48	3/6	91
(18)	C-c-1	—	38–45	41	—	—	13/14	6	53	7/7	117
(19)	C-c-1	CO_2 (9 times)	45–69	55	14	34%	12/14	8	47	3/4	94
(20)	C-l-1	—	38–45	42	—	—	13/15	5	59	7/8	116
(21)	C-l-1	CO_2 (11 times)	45–82	60	18	43%	13/15	5	45	7/8	81
(22)	C-c-5	—	36–46	41	—	—	14/16	9	49	4/5	110
(23)	C-c-5	CO_2 (11 times)	46–70	54	13	32%	15/16	5	47	8/10	90
(24)	C-l-5	—	42–53	47	—	—	10/16	5	50	1/5	105
(25)	C-l-5	CO_2 (13 times)	52–89	67	20	43%	16/16	10	48	2/6	76
(26)	C-c-10	—	38–46	43	—	—	15/16	6	48	5/9	101
(27)	C-c-10	CO_2 (12 times)	49–78	56	13	30%	14/16	8	48	3/6	89
(28)	C-l-10	—	50–63	57	—	—	10/16	5	51	1/5	89
(29)	C-l-10	CO_2 (14 times)	56–102	75	18	31%	14/16	11	52	1/3	90
(30)	C-c-20	—	44–82	52	—	—	12/16	6	52	2/6	87
(31)	C-c-20	CO_2 (11 times)	56–79	67	15	29%	13/16	7	44	2/6	73
(32)	C-l-20	—	78–106	90	—	—	7/16	3	48	1/4	74
(33)	C-l-20	CO_2 (16 times)	96–113	105	15	17%	7/16	4	44	0/3	70

* DB = dog biscuit ; C = casein diet with c = corn oil, and l = linoleic acid, 1, 5, 10, or 20% as indicated by numerals.

† The numerator is the number of insects which lived to maturity while the denominator is the number of nymphs at start of experiment.

‡ The numerator is the number of females with extruded oöthecae while the denominator is the total number of adult females.

Later, groups of various numbers of nymphs were put into the pint jars and reared in the temperature cabinet. According to expectations, the nymphs reared in isolation grew more slowly (10 days longer) than those reared with companions. Consistent with previous results, the CO_2 retarded growth by 20 days or 39% for isolated nymphs and 19 days or 46% for groups of 2 or more. In other words, the anaesthetic retarded the roaches more than did rearing in isolation, but it also imposed an additional retardation on isolated ones. Therefore, CO_2 did not reduce the growth response of grouped roaches to that of isolated ones.

Contrasted with the results of short exposure to CO_2, on several occasions groups of nymphs were kept anaesthetized for 2½ hours with a fluctuating CO_2 concentration. The following day these insects were enormously distended with gas, and if pricked with a needle they collapsed like a rubber balloon. They remained more or less stupefied and paralysed until they died several days later. Nitrogen, on the other hand, is a much less effective anaesthetic. It requires minutes instead of seconds for knockdown, and is followed by rapid and spontaneous recovery. When nymphs were kept anaesthetized with N_2 for 2½ hours, there was distension of the abdomens, but by the next day the insects had all recovered.

DISCUSSION

While growth-retardation as a result of repeated carbon-dioxide anaesthesia was consistently observed, the variations in amount of retardation could not be correlated with the diets which were tested, and there was no other indication as to how this effect is mediated. These facts should serve as a warning against the regular use of carbon-dioxide anaesthesia of cockroaches on experimental diets.

LEVENBOOK (1950a) has pointed out that there are five forms in which carbon dioxide may be transported in blood. The first of these is the dissolved anhydrous gas, CO_2. Because of solubility considerations, the amount of blood is not sufficient to account for the large volume percentage of CO_2 in the dissolved form in the blood of those insects for which data are available.

The second form is carbonic acid, H_2CO_3, but the dissociation constant is such that this acid is formed to the extent of only about 1%. Although the reaction $H_2O+CO_2 \rightleftharpoons H_2CO_3$ is catalysed by carbonic anhydrase, this enzyme has not been reported for the haemolymph of insects other than *Chironomus*.

In the presence of free bases such as Na^+, Ca^{++}, K^+, and Mg^{++} at pH 8 and above, significant amounts are formed of the third form, bicarbonate salts, $BHCO_3$. But LEVENBOOK emphasizes that at the physiological pH of insect blood (pH 6–7), bicarbonates are practically nonexistent.

The fourth form is the carbamate, R.NH.COOH, formed when CO_2 reacts with the free $-NH_2$ group of a protein, amino acid or amine. This reaction is rapid and spontaneous, and is promoted by a decrease in temperature and a pH of increasing alkalinity, but, according to LEVENBOOK, its physiological importance is controversial.

The final form is Y-bound-CO_2, and it is LEVENBOOK's opinion that gaseous CO_2 produced by insect tissues is bound in some form in the blood before its reconversion into the gaseous state and subsequent removal by diffusion.

LEVENBOOK (1950b) further expressed the opinion that the U-shape curve for buffering capacity of insect blood may be related to the inefficiency of the tracheal respiratory system in eliminating CO_2.

L'HÉRITIER (1948) has also suggested that the ability of *Drosophila* to become conditioned to CO_2 may be the result of the binding of CO_2 with a chemical precursor of the toxic process, the reaction being reversible.

In regard to the work reported in the present paper, the assumption may be made that the fate of gaseous CO_2 entering the blood from the external environment through the tracheal system may be the same as CO_2 entering from the tissues. If this assumption is permissible, then, according to LEVENBOOK's hypothesis, the externally applied CO_2 may be rapidly bound to some substance in the blood where it is retained because of inefficient elimination processes.

Based on the evidence that N_2 acts as an anaesthetic without lethal or growth-retarding effects, the bound-CO_2 hypothesis seems more likely than that the effect is due to a lack of O_2. The question then centres about the nature of the toxic action, but no more can be said at the present time than that a definite toxic effect is demonstrable.

It is a pleasure to acknowledge the helpful suggestions of Dr. A. GLENN RICHARDS during this work.

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THE MEDIAN NERVES AND COCKROACH SPIRACULAR FUNCTION

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Abstract—The median nerves of *Periplaneta americana* (L.) and *Blaberus craniifer* Burm. have been shown to control participation of the spiracles in the active ventilation process. Electrophysiological evidence indicates that each thoracic median nerve contains two axones which bifurcate to send branches to right and left spiracular muscles. The only sensory imput from the spiracular region appears to be from hair mechanoreceptors, stimulation of which causes closure of both spiracles at that level. Section of the median n. reduces CO_2 sensitivity of the spiracle and, after several days, results in fasciculation of the spiracle muscle. Section of the median n. does not affect electrical sensitivity of the spiracle muscle for at least 3 months. Regeneration of the median n. may occur in adult roaches and is accompanied by cessation of fasciculation and a return to normal activity on the part of the affected spiracle. The gross morphology of the thoracic and abdominal median nerves is briefly described.

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INTRODUCTION

INSECT spiracles often play a more complex rôle in respiration than simply as valves controlling passive gas diffusion. For example, cockroach spiracles open and close during active ventilation in a sequence synchronized with pumping movements produced by the thoracic and abdominal musculature (KITCHEL and HOSKINS, 1935). The resultant one-way air flow through the major tracheae serves greatly to reduce the respiratory dead-space.

Control of this complex ventilating process in insects is generally assumed to originate within the central nervous system (STAHN, 1928; FRAENKEL, 1932) which does, in fact, manifest spontaneous, rhythmic electrical activity similar in tempo to the respiratory cycle (ADRIAN, 1931; PROSSER, 1936). However, neural pathways connecting spiracles with the central nervous system have never been demonstrated experimentally, despite the fact that anatomical information suggests that the median nerves might supply the neural link (SNODGRASS, 1935; SCHMITT, 1954; BECKEL, 1955; and others). In the present investigation of the median nerves of the cockroach, this suggestion has been explored and confirmed. Some of the results have appeared in a preliminary report (CASE, 1956b).

MATERIAL AND METHODS

Adult male *Periplaneta americana* (L.) and *Blaberus craniifer* Burm., reared in mass culture, were used, males in preference to females because of their smaller fat deposits. *P. americana* was used for all anatomical observations and nerve defect experiments while both species were employed in neurophysiological studies.

Gross anatomical observations at magnifications up to 80 diameters were made on fresh specimens dissected in 0·9% NaCl after supravital staining with acetic nigrosine or aqueous 0·2% methylene blue. Teased preparations were examined at higher magnifications after dehydrating and clearing.

Nerve sections and other operations were performed, without vital staining, upon roaches anaesthetized with CO_2, utilizing a ventral approach. Only large incisions were sealed. Molten beeswax was used for sealing. After operation the roaches were isolated in Petri dishes and supplied with water and dog biscuit.

Tests of spiracle function were made by exposing roaches to mixtures of CO_2, O_2 and N_2, produced as previously described (CASE, 1956a), in a small observation chamber fitted to the stage of a dissecting microscope. In this chamber roaches were restrained with modelling clay so that an operated spiracle and its normal control opposite could be observed simultaneously. Subsequent to these tests neural damage was evaluated by dissection.

The electrical analysis of neural pathways was performed on decapitated roaches, usually prepared by dorsal approach involving removal of the gut and irrigation at intervals with HOYLE's (1955) basic locust saline. Electrodes were 40mμ diameter silver or 5 mil platinum hooks insulated with Formvar except for the inner hook surface. Hooks were placed in position with Brinkmann manipulators. Electrical impulses were conducted to Offner 140-A differential amplifiers and then to a Dumont dual-beam cathode-ray oscillograph. In some experiments spiracular movements were recorded by displacement of a fine glass stylus which actuated a piezoelectric phonograph pickup (ROEDER and WEIANT, 1950). A Grass S–4–B stimulator was used.

Mineral oil could be used effectively on *Blaberus* preparations although it caused rapid deterioration in *Periplaneta*.

RESULTS

A. *Description of spiracle innervation*

1. *Thoracic spiracles and median nerves.* The pattern of innervation of the second thoracic spiracle, typical also of the first, is shown in Fig. 1. Here the ganglion of the mesothoracic segment (Th II) is uppermost with the metathoracic segmental ganglion (Th III) directly below. Major nerves leaving Th II are numbered in anterio-dorsal to posterio-ventral sequence. A superficial dissection which serves to indicate nerve pathways in relation to some of the prominent muscles of the region is represented by the right-hand portion of the diagram where muscles are numbered according to the system of CARBONELL (1947). Thus the occlusor muscle of the second thoracic spiracle is m. 147.

It is evident from Fig. 1 that the second spiracle appears to be innervated from two sources, namely a branch of the median nerve of Th II, henceforth to be called transverse n., and branches of n. 1 of Th III. Supravital methylene blue preparations of Th II median n. rather clearly show two intensely stained nerve fibres which bifurcate, each sending a process down each transverse n. Although these processes have not been traced the entire length of the transverse n., teased

preparations of m. 147 show that probably only two fibres enter the muscle. These subsequently give off branches which ramify over the entire muscle. Since the fibres contributed by n. 1 Th III to the transverse n. leave it before insertion

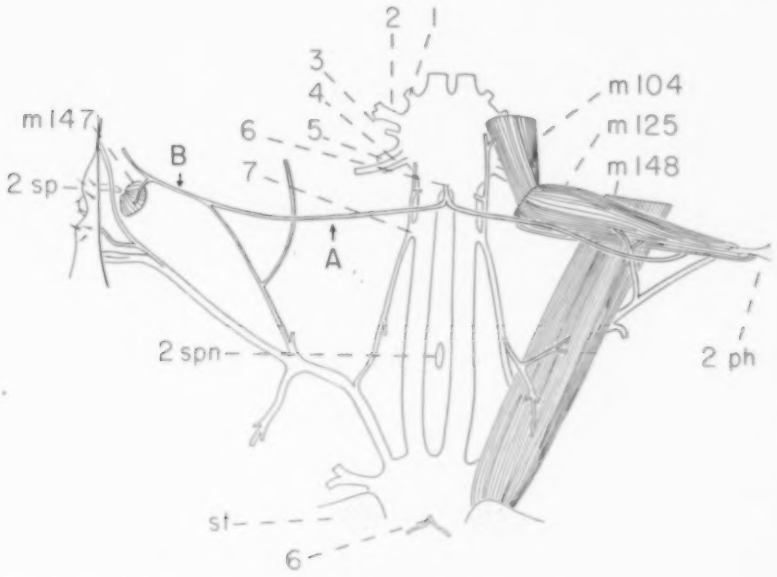

FIG. 1. Ventral dissection of *Periplaneta americana* illustrating the innervation of the second thoracic spiracle. Note median n., n. 6, leaving mesothoracic (Th II) ganglion to immediately bifurcate into left and right transverse nerves. (sp) spiracle; (spn) spina; (ph) phragma; (st) sternal arm; (A and B) electrode positions (see text).

on the muscle, the morphological evidence is in favour of the existence of a rather unusual situation, one in which a motor neuron provides innervation for two separate muscles lying on opposite sides of the body. The electrophysiological observations to be discussed below support this conclusion.

The several branches of n. 1 Th III found in the spiracular area may be traced into the hypodermis where some innervate hair sensillae.

2. *Abdominal spiracles and median nerves.* Fig. 2 delineates the relationship between the median nerves and abdominal spiracles. Two differences between the thoracic and abdominal median nerve systems are immediately apparent: the abdominal median nerves have segmental interconnections external to the ventral nerve cord whereas the median nerves of the thorax are not so interconnected; and there are thickenings in the abdominal median nerves which are probably ganglia.

The abdominal system of median nerves originates in Th III where two median nerves arise, probably a reflection of the composite origin of this ganglion from at least two embryonic ganglia. The anterior member of the pair evidently bifurcates before emerging from the ganglion so that all that is seen is a pair of

transverse nerves extending to the dorsally situated first abdominal spiracles (3 sp, Fig. 2). The posterior median n. of Th III emerges from the left connective and passes in the ventral diaphragm to the ventral surface of the first

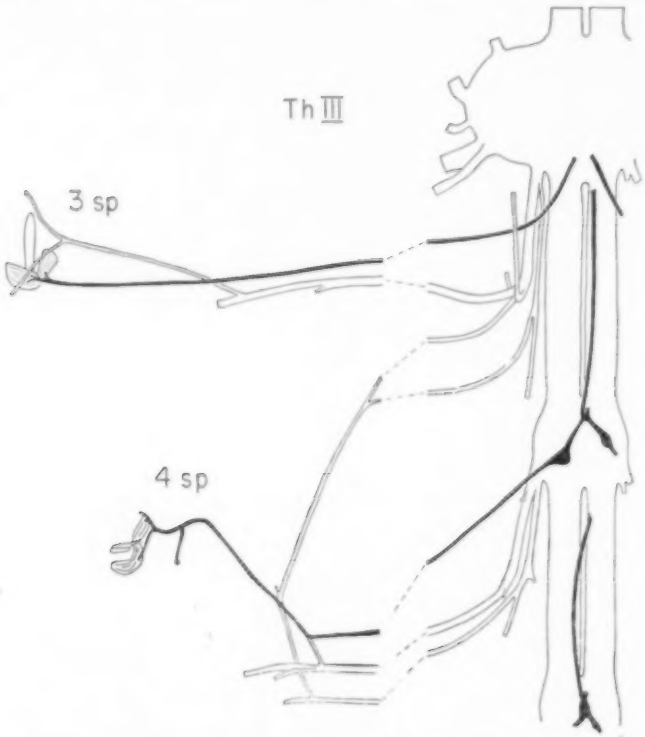

FIG. 2. Ventral dissection of *Periplaneta americana* illustrating the innervation of the first and second abdominal spiracles. Median nerves in black. Dotted lines indicate horizontal foreshortening.

abdominal ganglion (Abd I) to connect with the median n. of that ganglion. Similar arrangements are repeated along the cord until the terminal ganglion is reached. Here the median n. extending to the terminal ganglion from the penultimate ganglion makes no external connection with any of the three median nerves issuing from the terminal ganglion but fuses with the connective. The most anterior of the median nerves of the terminal ganglion is concerned with the innervation of the last abdominal spiracle. The posterior pair is fused with the cercal nerves.

The presumptive ganglia of the abdominal median nerves remain to be examined in section. They have, at present, been studied as whole mounts from which it was possible only to ascertain that they contain several nuclei.

Despite these variations, similarities are evident in the patterns of innervation of both thoracic and abdominal spiracles. We have seen that thoracic spiracles

are supplied with nerves from two ganglia of the central nervous system. Similarly, in the case of a typical abdominal spiracle, for example (Fig. 2, 4 sp), the innervation of the opening and closing muscles is again compound, appearing grossly to come from the median n. of Abd I as well as the ventral nerves of Th III and Abd I. Only the first and last abdominal spiracles seem to violate this pattern of intersegmental spiracle innervation, and the violation in these instances may be more apparent than real in the light of the composite nature of the last thoracic and last abdominal ganglia.

B. *Experimental analysis of spiracle innervation*

1. *Neural defects.* These experiments, performed for convenience mostly upon the second thoracic spiracle of *P. americana*, demonstrate the importance of the median nerves to participation of the spiracles in the ventilating process. In more than 200 instances, sectioning or crushing a transverse or median nerve, thoracic or abdominal, always rendered the denervated spiracles non-functional during active ventilation. Transsection of the ventral nerve cord was the only other neural intervention which affected rhythmic action of the spiracles. After isolation of Th II from the remainder of the central nervous system by cord transsections, the second thoracic spiracle pair continued to function rhythmically during active ventilation; but this rhythm was not necessarily in phase with that of the first thoracic spiracles. This is taken to be evidence of an integrative mechanism operative between successive spiracle pairs.

Immediately upon transverse or median nerve section the affected spiracles opened and remained so for several hours. Within 24 hours of sectioning, these spiracles were found to have become entirely quiescent and to have shut. By this time they had also become grossly insensitive to CO_2. More than 15% CO_2 in 20% O_2 was necessary to induce opening compared with the 2 to 5% required for normal spiracles. Denervated spiracles did not open even during spontaneous, active ventilation. During the first week after denervation these spiracles, once induced to open by high CO_2 concentrations, lagged far behind their normal mates in closing. During this period the contractions of the denervated occlusor muscles were relatively fast twitches which involved the entire muscle.

Four to six days after transverse or median n. section a radical change in the quality of spiracle muscle contractions occurred. By this time the muscle began to fasciculate, that is, to undergo nearly continuous, slow contractions and relaxations of small groups of muscle fibres. This phenomenon is here termed fasciculation, rather than fibrillation, because the unit of activity appears to be larger than the individual muscle cell. The contractions produced in this manner are more than strong enough to close the spiracle, and, with their onset, spiracles began to close more promptly after CO_2 induced opening although there was no evident change in CO_2 sensitivity. Fasciculation was more or less continuous. When for any reason it momentarily ceased, it could be started again by lightly tapping the cuticle overlying the muscle. That this phenomenon is probably the result of loss of the peripheral median n. stump seems likely. Certainly the early onset of

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fasciculation is evidence that it is not the result of re-entry of nerves into the muscle, and this is further attested by persistence of fasciculation for some time *in vitro*.

Regeneration of the transverse nerve occurred frequently. Of those individuals which had had only a transverse nerve sectioned, normal function returned in only 18 (36%) after an interval of between 16 and 32 days. A few unrecovered individuals which were followed for longer than 3 months showed no cessation of fasciculation. Cutting both the transverse n. and n. 1 resulted in recovery of 15 animals (33%) in 25 to 40 days. The longer recovery interval most likely reflects the more extensive trauma associated with this operation as compared with section of the transverse nerve alone. Merely crushing the transverse n. between the tips of watchmakers' forceps was always followed by recovery (11 insects) within 9 to 15 days. The return to normal in every instance was accompanied by cessation of fasciculation and return to whole-muscle, fast twitches. Dissection of recovered specimens revealed clear evidence of re-innervation of the spiracle muscle by outgrowth of the median n.

2. *Electrical studies.* In Fig. 3 are shown a diagram of the experimental arrangement for recording from the transverse n. in *P. americana* and examples of characteristic oscillograph traces obtained from such preparations. The upper strip demonstrates the correlation between movements of the spiracle and impulses in the nerve. Here the upper beam registers the output of a piezoelectric phonograph cartridge actuated by a glass fibre stylus placed among some of the stout hairs at the base of the spiracle lip. The characteristics of pickup and amplifier were such that only the initiation of a spiracle movement was registered. The lower beam, upper strip, registers electrical activity in the intact transverse n. In this experiment spiracle closures were commencing after CO_2-induced hyperventilation, and it may seem that the most intense electrical activity occurred when the spiracle began to move. The lower strip represents transverse n. activity obtained after distal section which permitted raising the preparation somewhat higher out of the body fluids. The greater part of two volleys is seen, and there appear to be two active motor fibres. Similarly, there seem to be only two motor fibres in the *Blaberus* thoracic transverse n. (Fig. 4). At this level, approximately point A in Fig. 1, only motor activity has been so far detected in the transverse n. in both species; after section or crushing proximal to the electrodes the nerve becomes electrically silent. In *Blaberus*, recordings from point B in Fig. 1 show that all motor activity is derived from the median n. The only sensory activity recorded at this point has been from mechanoreceptors, all of which enter the central nervous system *via* n. 1. No afferent activity was detected in either the median n. or n. 1 as a consequence of exposure of the spiracular region to low concentrations of CO_2. Higher CO_2 concentrations elicited considerable activity in n. 1, but this appears to have been derived entirely from mechanoreceptors activated by CO_2-induced muscle tremors.

Except for the transverse and median nerves, electrical stimulation of any of the nerves in the vicinity of the thoracic spiracles had no apparent effect on the

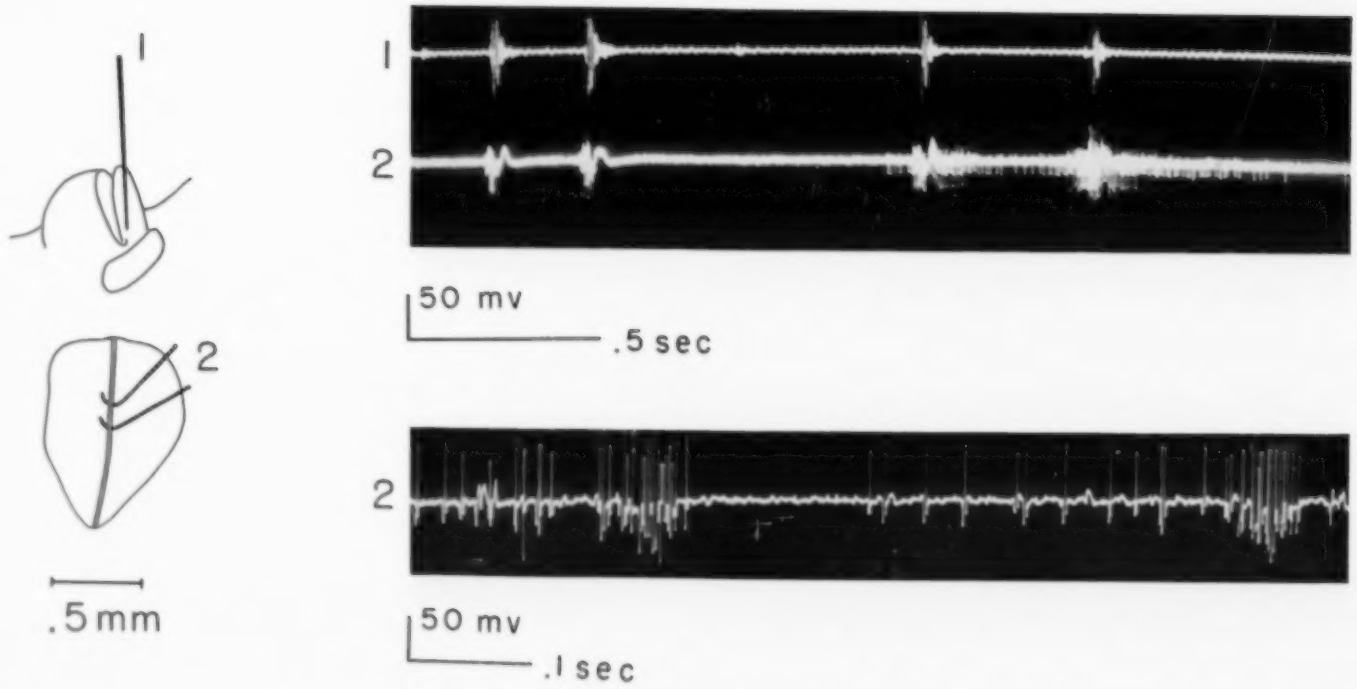

FIG. 3. Correlation between electrical impulses in the transverse n. and movements of the second thoracic spiracle in *Periplaneta americana*. Diagram shows recording arrangement: (1) glass fibre to phonograph cartridge; (2) recording electrodes.

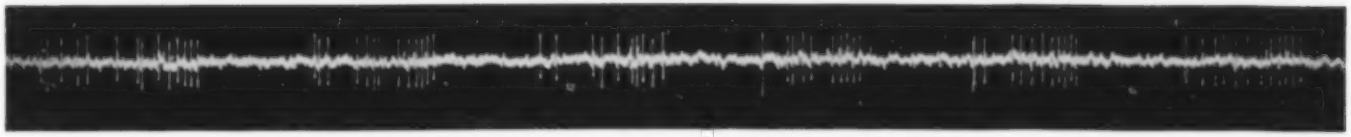

FIG. 4. Rhythmic impulses in transverse n. of *Blaberus craniifer* during spontaneous hyperventilation. Two fibres are active. Bipolar recording. Vertical line, 50 mV; horizontal line, 1 sec.

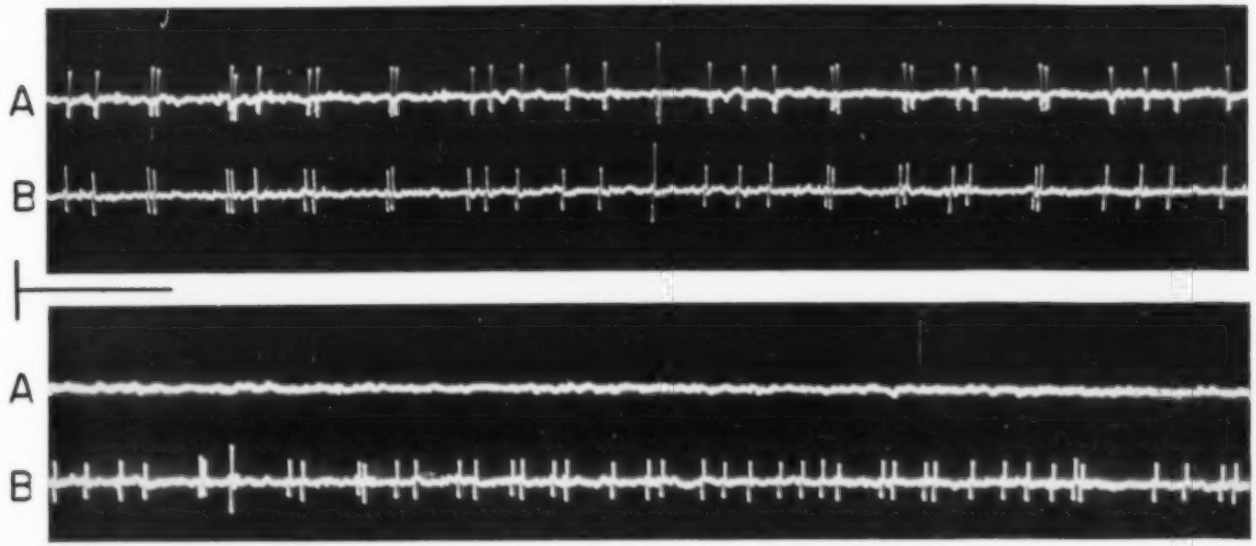

FIG. 5. Synchronous impulses in right (A) and left (B) transverse nerves of the second thoracic median n. of *Blaberus craniifer*. In the lower strip, the right transverse n. has been crushed proximally to the electrodes. Bipolar recording. Vertical line, 50 mV; horizontal line, 1·0 sec.

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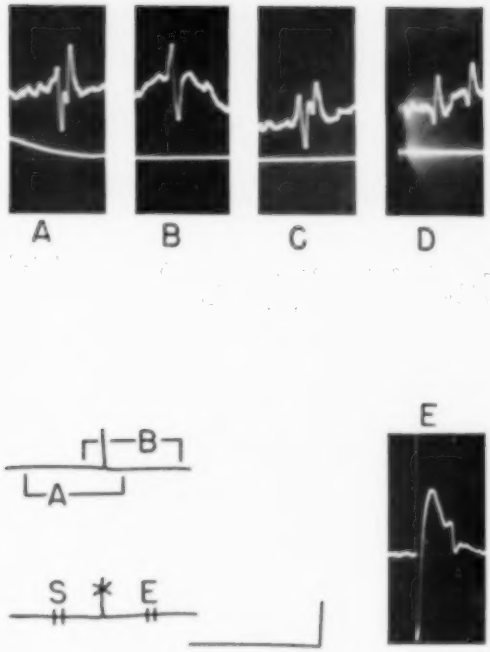

FIG. 6. A–D, effect of shifting position of electrode pair on apparent sign of spontaneous impulses in transverse nerves. (A and B) with electrode positions as shown in upper diagram; (C) after return of electrodes to position A; (D) monophasic potentials recorded after crushing one transverse n. proximal to electrode; (E) transmission of impulse from left to right median n. after crushing median n. between bifurcation and Th II. Recording arrangement shown in lower diagram: (S) point of stimulation (0·4 V, 0·1 ms); (E) bipolar recording electrodes; (X) point of crushing. Vertical line, 50 mV; horizontal line, 5 ms.

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spiracle muscles. Stimulation of the transverse n. induced only closure. Immediately after section of the transverse n., visible twitches were elicited from the denervated spiracle muscle by direct electrical stimulation or *via* the peripheral stump of the transverse n. Responses of the muscle to direct electrical stimulation were evidently fairly normal some 2 months after transverse n. section. Chronaxie and rheobase of normal and denervated muscles were not appreciably different, although it must be admitted that the end points in these determinations were based only on observation of twitches of the most sensitive fibres at 40× magnification, rather than upon electrical responses. The major significance of these observations lies in the fact that they demonstrate the fasciculating muscle to be responsive to electrical stimulation and indicate the denervated spiracle muscle may possibly remain electrically excitable indefinitely.

The primary importance of the median nerve to centrally co-ordinated spiracle movements is again illustrated by a closing reflex elicited in both spiracles of a given thoracic pair by touching one of many hair mechanoreceptors of one member of the pair. Section of the transverse n. to one spiracle stopped its own closing response but had no effect on the opposite spiracle. The afferent reflex component travels centrally along n. 1, thence to the next anterior ganglion by either connective, as was shown by cord hemisection, and from there to the spiracle muscle *via* the median n. of that ganglion. With both transverse elements of the median n. intact, reflexly stimulated closures of both spiracles occurred in exact synchrony as far as could be judged by eye.

It will be recalled from earlier discussion that morphological evidence exists for axonal bifurcation in the thoracic median nerves. Support is lent to this contention by the following observations. Recordings from right and left transverse branches of the median n. of both *Periplaneta* and *Blaberus* revealed exactly synchronous nerve impulses (Fig. 5). This synchrony was confirmed when the initial apparent sign of the spike potential was observed as a function of the position along the transverse nerves of an electrode pair (Fig. 6). If impulses are nearly simultaneously moving peripherally from the point of bifurcation, the first electrode to detect the impulse will be the one nearest the bifurcation. The apparent initial sign of impulses detected in this position will be opposite to that occurring when the second electrode is nearest the junction. The second deflection in the oscillograph trace is, in each case, produced by impulse passage across the more distant electrode.

It is true that synchrony could as well be produced by a central integrative mechanism which ensured simultaneous firing of two separate neurons whose axones progressed independently down each transverse branch. However, since an impulse moving centrally in one of the transverse branches crosses to the other, even when the median n. has been sectioned (Fig. 6), this mechanism does not necessarily exist.

DISCUSSION

The morphological and experimental observations reported here constitute conclusive evidence of the essential rôle of the median nerves in normal function of the spiracles of roaches. While the complex behaviour of the spiracles during

active ventilation alone would argue powerfully for their central regulation, this argument is supported decisively by morphological demonstration of a possible neural pathway and by experimental proof of the function of this pathway.

Historically, the median nerves have long been suspected of a respiratory function. In 1834 they were styled by NEWPORT as "The Transverse, Superadded, or Respiratory Nerves" (NEWPORT, 1834, p. 401) because of their evident termination near the spiracles. Subsequent authors have confirmed and extended his observations to other species, but apparently not until SWAINE's (1920) description of the central nervous system of *Sthenopis thule* were the median nerves followed into the spiracle muscles. SCHMITT (1954) appears to have made the only previous anatomical observations on the median nerves of roaches. The present study is in agreement with his findings, which concern the innervation of the pregenital abdominal segments, except for his belief that there is no intersegmental median n. trunk in *Periplaneta*. This structure is assuredly present, albeit frequently difficult to observe because of its intimate association with the ventral diaphragm and because of the fatty deposits which occur on the ventral nerve cord.

ZAWARZIN (1924) described in the *Aeschna* larva apparent instances of axonal bifurcation in the median n. His observations are here largely confirmed, as far as thoracic and terminal abdominal ganglia are concerned, except that there is no electrophysiological evidence of sensory fibres in the median nerves. While this may represent only a species difference, one might also argue that ZAWARZIN's histological techniques could not actually differentiate motor and sensory fibres.

During the years since their discovery the median nerves have remained almost completely unknown physiologically. In recent years only two authors have discussed their function. ZAWARZIN (1916) reasoned that they were elements of an insectan vegetative nervous system. Because his thesis rests only on a dubious analogy with the vertebrate central nervous system further comment is unnecessary. Somewhat more tangible is the related proposal of VOSKRESENSKAYA (1950) who, again, looked upon these nerves as analogues of the vertebrate sympathetic nervous system and who based her belief, not only upon the structural analogy, but upon experiments tending to show an influence of these nerves on the irritability of the flight muscles of the migratory locust. Neither of these investigations were concerned with a respiratory function for the median nerves.

Spontaneous muscular activity consequent upon loss of motor innervation appears to be a phenomenon new to the literature of insect physiology although it is common enough among vertebrates. HOYLE (1955) has reported the occurrence of fibrillation (random spontaneous contractions of individual muscle fibrils) in the locust extensor tibialis upon exposure to high concentrations of magnesium. This has been confirmed in *Periplaneta*. Because the spontaneous muscular activity induced by denervation seems to involve muscle units larger than those concerned in magnesium-induced fibrillation, it was felt that a different descriptive term was required for it. Fasciculation is probably appropriate since, with reference to the vertebrate, it is frequently used to denote spontaneous activity of muscle fascicules or motor units (*e.g.* ADAMS *et al.*, 1953, p. 90).

Perhaps the most significant problem touched upon in this investigation is that of the propagated muscle potential, the occurrence of which in insects has been questioned by ROEDER and WEIANT (1950). In their experiments, denervation of the tergal muscles of the metathoracic trochantin in *Periplaneta* was followed in 3 to 5 days by complete electrical inexcitability, suggesting ". . . that the muscle can be excited only through the agency of the motor nerve impulse" (*loc. cit.*, p. 9). Their observation is confirmed in a recent investigation by BODENSTEIN (1956) who reported electrical inexcitability a few days after denervation of metathoracic coxal muscles in *Periplaneta* and who found, in addition, subsequent regeneration of the motor nerves to the coxal muscles accompanied by recovery of electrical excitability. WILSON (1954) has criticized this viewpoint on the grounds that denervation is apt to induce changes in muscle excitability, a criticism which is now considerably blunted by BODENSTEIN's report that a return to normal electrical excitability is associated with re-innervation. Granting this, WILSON still presents a cogent argument for propagated muscle potentials which is based on the observation, during intracellular recording from the flexor tibiae, of an overshoot exceeding the resting potential and of the occurrence of a refractory period following the action potential. Fasciculation and maintenance of normal electrical excitability following denervation of roach spiracle muscles certainly tends to support this argument for propagated muscle potentials for it is not easy to perceive how these phenomena could occur otherwise.

The location of CO_2-sensing elements in or near the spiracles, as has been proposed by HAZELHOFF (1926) for *Periplaneta* and CASE (1956a) for *Musca*, is left somewhat in doubt by the absence of electrical signs of chemoreceptor activity in either the transverse n. or n. 1 in both *Periplaneta* and *Blaberus*. Assuming this to be sufficient evidence that spiracular chemoreceptors are absent, there conceivably could be a direct inhibitory effect of CO_2 upon the peripheral regions of the transverse nerve which would cause the spiracle to open upon direct, local application of CO_2. Such a mechanism, if it does exist, is probably of no great physiological importance to the cockroach because it requires the CO_2-sensing component of the respiratory system to be located in the worst possible situation, that is, almost outside the insect where it would be most poorly located to monitor efficiently the respiratory requirements of more interior organs. Perhaps the search for insectan respiratory chemoreceptors could profitably be turned to the central nervous system which maintains rhythmic respiratory-like impulses after complete isolation from peripheral nerves (see introduction), which rhythmic activity is readily influenced by the ambient CO_2 tension (ROEDER, 1953) and O_2 tension (CASE and HASSETT, unpubl.).

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ÉTUDE DES MODIFICATIONS DE CERTAINES PROPRIÉTÉS DES ŒUFS DE *BOMBYX MORI* L. DANS PLUSIEURS GENERATIONS SUCCESSIVES SELON L'ALIMENTATION

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Abstract—It is possible to modify some of the characteristics of the F_1 generation of *Bombyx mori* L. by suitable treatment of the parents. Experimental cultures were reared on autumn leaves of mulberry and controls on spring leaves. The effects on the parents of a diet of autumn leaves are perceptible in the F_1 generation when this generation is reared in the autumn. The average weight of the eggs laid by the parent differs appreciably from the controls, but for the F_1 generation it is the total weight of the eggs laid that exhibits the greatest difference from the control series. This effect does not appear in the F_1 generation if this generation is reared in the spring, *i.e.* at the normal time for the species. Determination of the characteristics of the F_1 generation reared in the autumn occurs during ovogenesis and during the first larval stage.

INTRODUCTION

Nous avons démontré l'existence, chez *Bombyx mori* (Legay, 1955), de modifications importantes pour certains caractères de la ponte des papillons, selon les conditions d'élevage auxquelles avaient été soumises leurs larves.

Ces caractères étaient essentiellement: le poids total des œufs de la ponte (P), le poids moyen d'un œuf de la ponte (p). Quant aux conditions d'élevage elles étaient: d'une part celles d'un élevage normal de printemps (série I), d'autre part celles d'un élevage d'automne, soit avec de la feuille de mûrier ordinaire (série III) soit avec de la feuille choisie (série IV).

La question que nous posions dans les conclusions de cet article pour un examen ultérieur était de savoir quels effets pouvaient accompagner dans la descendance et en particulier dans la première génération les modifications observées sur les pontes.

Divers travaux effectués de 1953 à 1955 apportent des éléments contribuant à fixer une réponse à cette question.

Protocole experimental

Les œufs que nous pouvions utiliser provenaient soit de la série I (printemps 1953), soit des séries III et IV (automne 1953).

Nous nous sommes limités au trois groupes d'expériences suivants (*cf.* tableau 1):

(1) D'une part nous avons élevé à l'automne 1954 les descendants des séries III et IV de 1953, toutes deux dans des conditions analogues à celles des parents de la

série III (c'est-à-dire avec de la feuille ordinaire); puis à l'automne 1955, toujours dans les mêmes conditions, une F_2 de la série IV; il n'a pas été possible d'élever la F_2 de la série III pour des raisons purement matérielles.

TABLEAU 1—NOMBRE DE PONTES ÉLEVÉES (*a*) ET NOMBRE DE PONTES OBTENUES (*b*) APRÈS REPRODUCTION POUR CHACUNE DES EXPÉRIENCES

Premier groupe d'expériences

Série expérimentale	Automne 1953 Parents	Automne 1954 F_1		Automne 1955 F_2	
	b	*a*	*b*	*a*	*b*
III	23	3	20		
IV	42	6	35	1	20

Deuxième groupe d'expériences

Série expérimentale	Printemps 1953 Parents	Printemps 1954 F_1		Printemps 1955 F_2	
	b	*a*	*b*	*a*	*b*
I	50	20	59	5	48

Troisième groupe d'expériences

Série expérimentale	Printemps 1954 F_1	
	a	*b*
I	20	59
III	14	85
IV	24	62

Nota: I = Élevages témoins du printemps.
III = Série issue d'un élevage d'automne (feuille ordinaire).
IV = Série issue d'un élevage d'automne (feuille choisie).

(2) D'autre part nous avons élevé au printemps 1954 la descendance de la série I de 1953, puis au printemps 1955 la F_2 de la même série.

(3) Enfin au printemps 1954, outre les témoins du printemps (série I) ont été élevés dans les mêmes conditions que ceux-ci des animaux F_1 des séries III et IV.

Ce sont donc surtout les résultats concernant la F_2 qui sont incomplets, mais nous pensons devoir les rapporter pour faciliter l'interprétation.

Tous les animaux élevés l'ont été par pontes isolées. Les pontes conservées pour l'élevage n'ont pas été choisies au hasard parmi les pontes obtenues à la génération précédente, sauf en ce qui concerne les témoins du printemps (c'est-à-dire la série I). Les pontes ont été choisies parmi celles présentant les caractères *p* et *P* supérieurs à la moyenne dans la série IV, inférieurs à la moyenne dans la série III. Mais nous verrons plus loin ce qu'il faut penser de l'importance du choix des géniteurs dans ce type d'expériences.

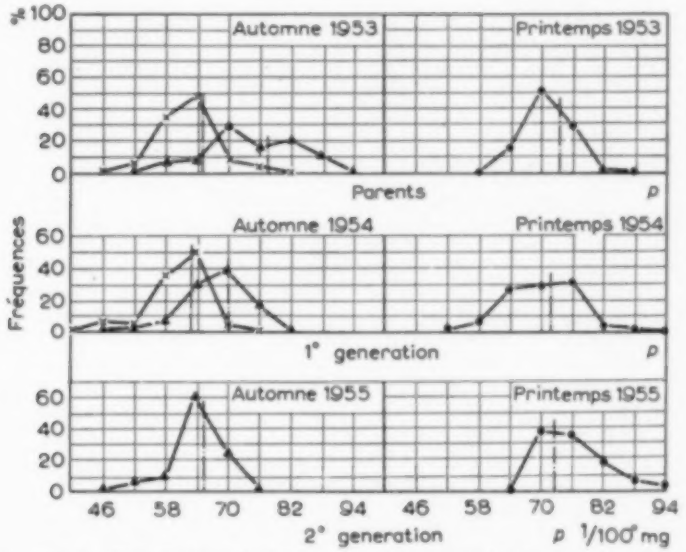
De plus, pour les élevages de 1954, ce sont dans la plupart des cas, les mêmes pontes qui ont été élevées au printemps et à l'automne, c'est-à-dire que les pontes ont été séparées en deux parties égales l'une étudiée au printemps, l'autre à l'automne. Les résultats obtenus autorisent donc des comparaisons d'autant plus valables.

Le tableau 1 résume le plan expérimental suivi.

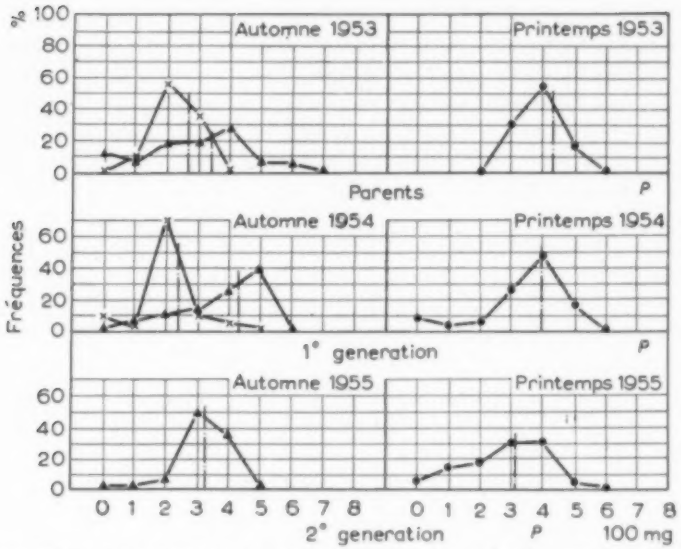
1ère PARTIE—ÉTUDE DES RÉSULTATS GLOBAUX CONCERNANT LES CARACTÈRES *p* ET *P*

1. *Résultats*

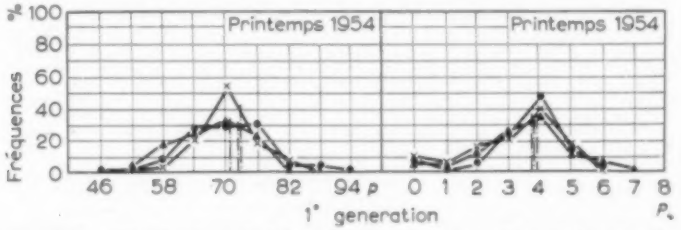
Les données numériques sont présentées ci-dessous de deux façons. D'une part les résultats globaux par séries expérimentales sous forme de moyennes (±erreur Standard, *cf.* tableau 2), d'autre part les répartitions des données individuelles sous forme de diagrammes de fréquences pour chaque série (*cf.* graphiques 1, 2, et 3).



GRAPHIQUE 1. Distributions de fréquences comparées selon les conditions expérimentales au cours de trois générations successives, pour le caractère *p* (poids moyen de l'œuf). ● série I: témoins élevés au printemps. ▲ série IV: animaux dont les parents ont été élevés à l'automne avec de la feuille choisie. × série III: animaux dont les parents ont été élevés à l'automne avec de la feuille ordinaire. —·—·—: position des moyennes.



GRAPHIQUE 2. Distributions de fréquences comparées selon les conditions expérimentales au cours de trois générations successives, pour le caractère *P* (poids de la ponte). ● série I: témoins élevés au printemps. ▲ série IV: animaux dont les parents ont été élevés à l'automne avec de la feuille choisie. × série III: animaux dont les parents ont été élevés à l'automne avec de la feuille ordinaire. —·—·—·: position des moyennes.



GRAPHIQUE 3. Distributions de fréquences comparées en F_1 pour les caractères *p* et *P*. ●: série I. ▲: série IV. ×: série III. —·—·—·: position des moyennes.

En ce qui concerne ce deuxième aspect, signalons que nous avons défini pour *p* des classes plus larges que dans une publication précédente (LEGAY, 1955), afin de faire pratiquement disparaître l'hétérogénéité dûe au dimorphisme pondéral habituel, simplifier les diagrammes, et obtenir des répartitions à peu près normales, ce qui facilite les comparaisons.

Nous avons séparé dans le tableau 2 comme dans les graphiques (1, 2, et 3) les résultats concernant les trois groupes d'expériences définis plus haut.

2. *Discussion—Interprétation*

Il y a lieu de remarquer dès l'abord que les résultats concernant le poids moyen d'un œuf (*p*) *pour les lots témoins* élevés au printemps 1953 (Parents), 1954

TABLEAU 2—VALEUR DES CARACTÈRES p ET P POUR LES DIFFÉRENTS TRAITEMENTS ET LES GÉNÉRATIONS SUCCESSIVES

Caractère	Série	Automne 1953	Automne 1954	Automne 1955
		Parents	F_1	F_2
p	III	0·655 ± 0·010	0·638 ± 0·012	
	IV	0·779 ± 0·014	0·709 ± 0·010	0·664 ± 0·010
P	III	271·6 ± 12	242·2 ± 19	
	IV	340·3 ± 24	430·3 ± 22	324·4 ± 16

Caractère	Série	Printemps 1953	Printemps 1954	Printemps 1955
		Parents	F_1	F_2
p	I	0·734 ± 0·007	0·724 ± 0·008	0·730 ± 0·008
P	I	435·6 ± 8	397·5 ± 16	317·5 ± 19

Caractère	Série	Printemps 1954
		F_1
p	I	0·724 ± 0·008
	III	0·727 ± 0·005
	IV	0·707 ± 0·007
P	I	397·5 ± 16
	III	379·3 ± 15
	IV	379·8 ± 19

Nota: I = Élevages témoins du printemps.
III = Série issue d'un élevage d'automne (feuille ordinaire).
IV = Série issue d'un élevage d'automne (feuille choisie).

(F_1), et 1955 (F_2) indiquent des valeurs stables,* d'une génération à l'autre et une variabilité constante. (La variabilité est d'ailleurs bien plus faible, en valeur relative, pour p que pour P, quelles que soient les séries expérimentales.) Par contre, pour ces mêmes lots témoins, les valeurs moyennes du poids de la ponte (P)

* Cette stabilité de p avait déjà été remarqué soit au cours d'expériences génétiques selon les lignées et les croisements (LEGAY, 1954), soit au cours d'expériences physiologiques ou l'agent modificateur de l'ovogénèse était la sous-alimentation quantitative (BAUD, 1955).

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baissent régulièrement, tandis que la variabilité augmente (*cf.* tableau 2 et graph. 2). Ces faits sont dus en grande part à un accroissement de la stérilité des œufs et de la mortalité embryonnaire, pour lesquelles on trouve les valeurs suivantes (exprimées en — % moyen d'œufs par ponte):

	Parents	F_1	F_2
Stérilité des œufs	0·1%	3·3%	9·8%
Mortalité embryonnaire	0·1%	1·8%	11·0%

Ces modifications ne gênent pas sensiblement l'interprétation en F_1, mais le feraient beaucoup plus en F_2. Elles peuvent être attribuées à l'imbreeding qu'il est matériellement difficile d'éviter dans ce type d'expériences, les croisements entre pontes n'étant pas toujours réalisables à cause des variations dans les dates d'apparition des papillons, et du peu de latitude que laissent les femelles pour la fécondation.

Quoiqu'il en soit, il est fort intéressant de noter que les effets de la consanguinité ne se font pas sentir de la même façon sur tous les caractères et que s'ils sont inexistants jusqu'en F_2 pour le poids moyen de l'œuf (p), ils sont très marqués pour le poids total (P) des œufs normaux des pontes. On peut conclure à un certain degré d'indépendance dans le déterminisme de ces deux caractères.

Ces mêmes modifications se manifestent sans doute dans les autres séries expérimentales, mais restent masquées par les variations principales qui sont précisément celles qui nous intéressaient au départ.

En ce qui concerne le poids moyen d'un œuf (p), *les deux séries III et IV*, différenciées à l'automne 1953 ont donné en F_1 à l'automne 1954, bien qu'élevées toutes les deux dans les mêmes conditions, des valeurs moyennes et des diagrammes de fréquences nettement distincts. La série III reste à peu près semblable à elle même (différence non significative entre parents et F_1); la série IV se rapproche de la série III, mais en reste distincte (différences significatives entre parents et F_1 de la série IV, mais aussi entre F_1 série III et F_1 série IV).

Ainsi donc les œufs issus de la F_1, qui représentent, rappelons-le, le début de la F_2, sont encore indiscutablement marqués par le traitement imposé à la génération parentale dans sa phase larvaire.

Les résultats partiels obtenus dans la série IV en F_2 (1955) avec des animaux issus d'une ponte, qui présentait pourtant les valeurs maximum de la série pour les caractères p et P, montrent que la différence induite entre les séries III et IV disparaitrait très probablement à cette génération.

En ce qui concerne pour les mêmes séries expérimentales (III et IV) le caractère P, nous observons en F_1 le phénomène contraire de celui qu'on observe pour p (*cf.* graph. 2). La moyenne du poids de la ponte (P) devient en effet très élevée, supérieure à celles de toutes les autres séries expérimentales y compris celles des témoins du printemps. Ainsi la différence induite entre les séries III

et IV, qui était à peine significative en 1953 en raison de la grande variabilité de P, le devient très nettement en F_1. La différence est également significative entre Parents et F_1 de la série IV.

Cette observation est à rapprocher de celle de WADDINGTON (1955) sur la Drosophile. Cet auteur est amené à subdiviser un caractère principal (nervation des ailes) en "sub-caractères", dont le déterminisme serait différent, pour expliquer les variations de sens opposé qui les touchent. Dans le cas de nos expériences il y a, à la fois, persistance en F_1 d'un effet qui, pour certains caractères, va en s'amenuisant comme c'est le cas pour p, mais aussi expression dans cette F_1 de la partie principale de l'effet pour d'autres caractères—comme c'est le cas pour P.

Si l'on tient compte que cette contradiction apparente dans les effets d'un même traitement disparait dans les générations ultérieures, il semble plus simple d'admettre un décalage dans les effets, décalage lié à la nature des relations entre p et P, comme nous le verrons plus loin. Ce décalage entre générations est encore décelable en F_2 (1955), pour laquelle la valeur de p (série IV) est redevenue comparable à celle des parents témoins (série III 1953) (différence non significative), alors que la valeur de P demeure sensiblement supérieure (différence significative).

Un autre aspect de nos résultats mérite attention. Nous avons vu que les F_1 de nos séries III et IV restent nettement dissemblables, si elles sont élevés à l'automne. Si, par contre, elles sont élevées au printemps, c'est-à-dire dans des conditions *différentes de celles des deux parents, mais comparables à celles de toutes les générations antérieures*, elles deviennent immédiatement semblables (*cf.* graph. 3) non seulement entre elles, mais encore avec la F_1 de la série I (c'est-à-dire le témoin du printemps). On peut interpréter ce fait de plusieurs façons.

On peut admettre que les conditions de l'élevage de printemps correspondent si fortement à l'adaptation normale de nos animaux qu'une seule génération suffit à effacer les différences établies entre nos séries, I, III, et IV, au cours de la vie larvaire de leurs parents.

Mais on peut également admettre que le regroupement constaté n'est qu'apparent et que les conditions standard d'élevage de printemps ne font que masquer une hétérogénéité sousjacente.

Pour choisir entre ces deux hypothèses, il faudrait élever et comparer les F_2 des différentes séries, non seulement à la suite d'une F_1 élevée à l'automne, mais aussi à la suite d'une F_1 élevée au printemps; ce qui n'a pas été réalisé.

Quelle que soit l'explication du phénomène, nous noterons *que selon les conditions d'élevage* le retour à un type donné est plus ou moins rapide. Autrement dit, la régulation qui intervient, c'est-à-dire ce retour à un niveau correspondant aux conditions de milieu habituelles est facilitée par ces conditions elles-mêmes, comme si le fonctionnement lié à un milieu marquait à la longue l'organisme et créait des ornières dans lesquelles retombent toujours les mécanismes, si ce milieu est rétabli. Il y a hérédité de la sensibilité à des conditions données. WADDINGTON (1942, 1955) avait déjà noté au cours de recherches d'embryologie l'importance des régulations permettant la naissance d'adultes relativement normaux, même si l'ontogénèse se déroulait dans des conditions plus ou moins anormales, il avait

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proposé le terme de "canalisation du développement" pour désigner ce phénomène. Les résultats exposés ici nous conduisent à une notion comparable.

Cette "canalisation" a sans aucun doute des bases physico-chimiques précises; elle peut correspondre à une sensibilisation. Bien que situées dans un contexte différent, des expériences récentes sur la Drosophile (DAVID, 1955), comportant la maintien pendant plusieurs générations de conditions inhabituelles mettent en évidence en F_1 une sensibilisation au facteur anormal, ce qui démontre la mise en jeu possible d'un tel mécanisme (qui jouerait vis à vis d'un facteur normal sous le nom d'adaptation).

De plus les résultats exposés plus haut font apparaître que le traitement imposé aux parents en 1953 était en fait à deux degrés: d'une part conditions générales d'un élevage d'automne, d'autre part dans ce nouveau milieu usage de feuilles de mûrier de qualités différentes. Ainsi, même lorsqu'on cherche à modifier des animaux par l'intermédiaire de leur alimentation, ce qui constitue pourtant un traitement ménagé par rapport à bien d'autres, on induit cependant des modifications complexes, on trouble des mécanismes divers ou le même mécanisme à des niveaux différents selon les conditions expérimentales, ce qui conduit à des effets très variés.

IIème PARTIE—ÉTUDE DU DÉVELOPPEMENT CONDUISANT À L'EXPRESSION DES CARACTÈRES *p* et *P*

Les résultats, tels qu'ils viennent d'être exposés et interprétés, concernent les caractères *p* et *P* considérés isolément et indépendamment du développement des animaux qui en sont porteurs.

Or, l'examen des caractéristiques pondérales des périodes larvaires, nymphales et imaginales d'une part, celui des valeurs relatives des caractères *p* et *P* d'autre part, permettent de compléter notre information de façon moins formelle.

C'est pourquoi nous avons rassemblé les documents suivants.

1. *Résultats numériques*

Nous indiquons au tableau 3 le poids moyen des chenilles aux différentes mues larvaires (les mesures ont été faites à la sortie de mue), le poids moyen des cocons (♂ et ♀ en proportions égales), le poids des papillons ♀ après la ponte (poids sec conditionné). Ces données sont fournies pour les séries expérimentales III et IV, pour les trois élevages de l'automne 1953, de l'automne 1954, et du printemps 1954.

Nous présentons par ailleurs quelques données pondérales concernant uniquement les individus femelles: cocons, coques, et pontes et la valeur relative de ces dernières par rapport aux premiers.

2. *Interprétation*

Notons d'abord que les différences pondérales induites par les traitements imposés aux séries III et IV à l'automne 1953 sont nettes dès la 1ère mue et s'amplifient jusqu'au cocon; elles se réduisent pour le poids du papillon. Ce résultat

TABLEAU 3—CARACTÉRISTIQUES PONDÉRALES DU DÉVELOPPEMENT DES ANIMAUX DES SÉRIES III ET IV

		1e mue	2e mue	3e mue	4e mue	Cocon ♂ + ♀	Papillon ♀ poids secs
Automne 1953	III	4·4	23·8	119·6	477·8	1061·0	55·6
	IV	6·0	34·8	170·5	797·0	1987·0	89·3
	$\frac{IV}{III}$	1·36	1·46	1·43	1·67	1·87	1·60
Automne 1954	III	3·8	25·9	118·0	564·4	1229·3	62·5
	IV	5·8	34·6	166·6	715·3	1892·6	90·4
	$\frac{IV}{III}$	1·52	1·34	1·41	1·27	1·54	1·45
Printemps 1954	III	5·3	30·3	171·5	793·2	2118·5	94·2
	IV	5·6	35·0	192·3	806·8	2129·0	98·7
	$\frac{IV}{III}$	1·06	1·15	1·12	1·01	1·00	1·04

indique que le gain de poids réalisé par les animaux de la série IV a été consacré plus à la ponte qu'à la carcasse des adultes.

De plus la différence de poids constaté sur papillon ou sur cocon ne se retrouve pas tel que sur les œufs dont le poids moyen est toujours plus près de la normale que celui de la femelle qui les a pondus. Il y a régulation à ce stade du développement.

Les différences se retrouvent dans les chenilles F_1 (automne 1954) nettes au premier âge puis s'atténuant progressivement.

Rappelons que les séries III et IV de la F_1 sont élevées dans les mêmes conditions à l'automne 1954; malgré ce, nous observons que les effets du traitement des parents restent très sensibles jusqu'à la fin de la 1ère génération.

Par contre en élevage de printemps ces effets sont très rapidement effacés. Si les caractéristiques pondérales de la série IV sont toujours supérieures à celles de la série III, elles le sont de façon peu marquée, même au 2ème âge qui est généralement le plus sensible.

Ainsi le 1er intermue, dont on sait qu'il est caractérisé par une intensité de croissance maximum, est également celui qui dans les conditions convenables se montre capable d'absorber les plus grandes inégalités de départ. Cette plasticité ne signifie pas que les modifications apportées dans un sens ou dans l'autre sont sans importance pour la suite du développement. Bien au contraire divers travaux concernant le déterminisme alimentaire des mues (LEGAY, 1955) ou la sécrétion de la soie (recherches japonaises) ont mis en évidence l'importance indiscutable des conditions dans lesquelles se déroule le 1er âge.

Nous pouvons considérer par ailleurs la fin du développement et les caractéristiques pondérales qui l'accompagnent dans le cas particulier des individus ♀; nous avons comparé sous cet angle les données numériques concernant, pour la F_1 des séries III et IV, au printemps et à l'automne 1954, les valeurs relatives du poids de la coque soyeuse et de la ponte rapportées au poids total du cocon. Nous notons deux faits importants.

Dans le cas de l'élevage de printemps aussi bien dans la série III que dans la série IV, les valeurs relatives de la coque soyeuse et de la ponte sont du même ordre. Nous avons d'ailleurs souvent vérifié ce fait dans les races françaises de vers à soie sur lesquelles nous avons expérimenté.

Dans le cas de l'élevage d'automne il n'en est pas du tout ainsi. Que ce soit pour la série III ou pour la série IV, on note une valeur relative de poids de la ponte beaucoup plus élevée que celle du poids de la coque. Mais en outre la valeur relative du poids de la ponte est pour une même série plus élevée à l'automne qu'au printemps.

Ce phénomène est très net dans le cas de la série IV. Il demeure indiscutable dans la série III et nous l'avons vérifié dans d'autres circonstances expérimentales.

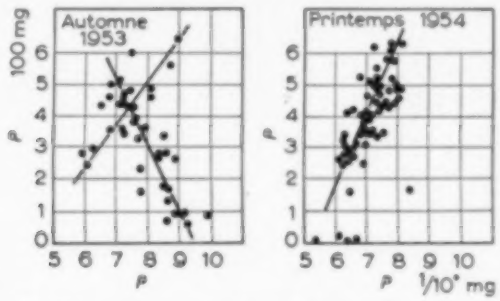
Les données de la série IV sont particulièrement intéressantes car les poids des cocons au printemps et à l'automne 1954 sont du même ordre. On constate en particulier que l'ensemble coque et ponte constitue un pourcentage identique du poids du cocon au printemps (33·3%) et à l'automne (33·9%) mais la répartition n'est pas la même: à l'automne la fécondité est plus élevée (20·1%) et la secrétion de soie plus faible (13·8%).

Sans préjuger des relations qui pourraient exister au stade individuel entre les caractéristiques quantitatives de l'ovogénèse et celles de la secrétion de la soie, nous constatons celles qui se manifestent pour un groupe d'individus selon les conditions expérimentales.

Si dans les conditions ordinaires d'automne, l'ovogénèse est modifiée, on peut penser que dans les conditions particulières d'élevage de la série IV à l'automne 1953, celle-ci a dû présenter des caractères qualitativement différents de ceux du printemps.

C'est ainsi que nous avons considéré les graphiques représentant les poids de ponte en fonction du poids moyen de l'œuf dans le cas de la série IV à l'automne 1953 et au printemps 1954 (*cf.* graph. 4). Nous notons l'opposition suivante: au printemps il y a proportionnalité entre les deux caractères P et p; à l'automne la situation est différente, au moins pour une grande partie des individus. Une corrélation inverse semble s'établir alors que pour les autres individus la proportionalité du printemps semble subsister. Les problèmes posés par ce graphique sont de deux ordres.

D'une part la signification de ce graphique double se pose. Il montre que dans certains cas, la relation entre p et P peut inverser sa tendance habituelle. Cette interprétation dualiste qui n'est pour le moment qu'une hypothèse appelle évidemment des recherches plus précises sur les bases mêmes de la relation entre p et P. Son intérêt serait d'aboutir à une explication du phénomène de décalage observé



GRAPHIQUE 4. Hypothèses concernant les relations entre les caractères p et P selon les conditions expérimentales.

dans les effets exprimés par les caractères p et P, en même temps que celui de "sensibilisation". Dans le cas de nos expériences il résulterait simplement du passage à une zone d'allométrie inversée, dont l'existence même pourrait être considérée *comme le moyen* d'une régulation.

D'autre part, devant l'importance des différences manifestées entre individus qu'il y a lieu de noter à l'examen des graphiques 4, on pouvait se demander si les résultats de la F_1 ne seraient pas modifiés selon que les géniteurs auraient été choisis dans la zone à p maximum et P minimum ou dans celle à P fort et p faible. La vérification—que nous n'avons pu faire malheureusement sur un grand nombre de pontes—semble indiquer que le choix des géniteurs importe peu sur les caractères de la F_1. La même constatation a été faite toujours dans la série IV en F_2 dont nous n'avons élevé qu'une ponte qui présentait pour p et P les valeurs maximum. Or cette F_2 a continué d'évoluer dans le sens général d'un retour aux caractères des parents, c'est à dire, dans le cas particulier d'une diminution des valeurs p et P.

Cette sorte de régulation se produit donc quels que soient les caractères propres des parents chosis dans la population expérimentée et intervient probablement de façon à ramener la valeur de ces caractères à des niveaux stables pour les conditions expérimentales choisies (*cf.* hypothèse de NIGON, 1954). Ces constatations conduisent à minimiser dans le cas de nos expériences le problème du choix des géniteurs, que nous avions posé à propos du protocole expérimental.

3. *Conclusions générales*

(1) Les effets du traitement alimentaire imposé aux parents restent sensibles en F_1, si l'élevage des animaux est réalisé à l'automne.

(2) Alors que chez les parents le poids moyen de l'œuf présentait les modifications les plus marquées, chez les enfants c'est le poids total des œufs de la ponte qui subit les plus fortes variations.

(3) Les effets disparaissent dès la F_1 si l'élevage des animaux est réalisé au printemps, c'est à dire dans les conditions habituelles de toutes les générations antérieures.

(4) La régulation qui se produit de toute façon à l'automne s'effectue essentiellement à deux stades principaux du développement: pendant l'ovogénèse et au cours du 1er intermue larvaire.

(5) Cette régulation se produit indépendamment des caractéristiques des géniteurs choisis dans la population parentale et pourrait s'effectuer par le moyen d'un changement d'allométrie.

L'intérêt de ces résultats réside entre autres dans le fait que les modifications des propriétés héréditaires concernant les œufs ont été obtenues par l'action d'une cause naturelle de variation: à savoir l'alimentation.

D'autre part les modifications constatées (en particulier augmentation du poids de la ponte en F_1) sont la preuve de la possibilité qui existe de modifier un caractère donné de la F_1 par un traitement approprié des parents. Le poids de l'œuf comme le poids de la ponte sont d'ailleurs des caractères dont l'intérêt pratique, bien que secondaire, est indiscutable.

Par ailleurs le point (4) de nos conclusions nous parait avoir une grande importance en génétique quantitative. Si, en effet, la plus grande part des régulations concernant un caractère d'adulte s'effectue à des stades jeunes ou embryonnaires, il est certain que les observations effectuées sur l'adulte ne peuvent être interprétées correctement qu'en tenant compte de l'existence de ces régulations, bien qu'il ne soit pas toujours facile de les mettre en évidence. Mais ceci suggère précisément de nombreuses expériences.

Enfin les modifications constatées, bien qu'on ne puisse saisir dans leur détail les mécanismes qui y conduisent, ne semblent nullement dénuées de signification générale. En effet le fait que deux générations successives présentent sous l'action du milieu, la première une fécondité très faible et la seconde une fécondité très forte constitue sans doute un des mécanismes intervenant chez les insectes sauvages dans le déterminisme des pullulations, et leur régulation.

Et cela d'autant plus que selon les circonstances écologiques beaucoup d'espèces présentent un nombre de générations variables par an, ce qui peut amener l'une d'elles à se développer à l'automne, c'est-à-dire dans des conditions analogues à celles que nous avons évoquées expérimentalement.

LIKVENTOV (1955) est arrivé récemment à une opinion comparable par des voies différentes. Il note sur *Lymantria dispar* la grande variabilité du poids des œufs et le problème que pose la relation entre la fécondité et poids moyen de l'œuf; il émet de plus l'hypothèse qu'une ponte importante à poids moyen d'œuf faible donnerait naissance à une génération affaiblie, sensible aux épizooties et susceptible d'extinction alors qu'une ponte faible à œufs lourds aboutira au résultat inverse et en définitive à un accroissement de la population. Nos expériences montrent qu'une telle hypothèse est très probablement acceptable dans certaines conditions.

En réalité, de nombreuses études ont montré les relations existant entre l'alimentation de la larve ou de l'adulte et la fécondité, mais très peu ont abordé expérimentalement l'aspect génétique du problème et n'ont en définitive formulé que des hypothèses.

TABLEAU 4—DONNÉES NUMÉRIQUES SUR LES COCONS, COQUES ET PONTES DES INDIVIDUS FEMELLES DES SÉRIES III ET IV

Séries expérimentales		Printemps 1954		Automne 1954		Automne 1955
		III	IV	III	IV	IV
en mg	Cocon	2285·7	2313·8	1349·4	2137·5	1573·8
	Coque	385·9	391·4	157·6	293·9	222·9
	Ponte	380·4	379·8	242·2	430·3	324·5
en % du Cocon	Coque	16·9%	16·9%	11·7%	13·8%	14·2%
	Ponte	16·6%	16·4%	17·9%	20·1%	20·6%
	Coque + Ponte		33·3%		33·9%	

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THE ELECTRICAL RESPONSES OF DORSAL OCELLI IN COCKROACHES AND GRASSHOPPERS*

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Abstract—The electroretinograms of dorsal ocelli in cockroaches and grasshoppers were recorded using extra-cellular electrodes. Different components of the electroretinograms were allocated to their sites of origin. Two slow potentials with opposite electrical sign, which sum algebraically and which may interact in an oscillatory manner, originate in the layer of photoreceptor cell bodies. Efferent nerve impulses, originating in the largest of the ocellar nerve fibres, appear on darkening the ocellus.

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INTRODUCTION

THIS paper is concerned with the analysis of electrical responses of insect dorsal ocelli. The chief objective is to identify that component of the electrical sequence which corresponds to the photoreceptor cell response. It is important to fulfil this objective if the easily recorded electrical responses are to be useful as indicators in the further study of basic photoreceptor cell processes.

The compound eye has been studied by a number of investigators, whose purpose has been the same, but there is little general agreement among them concerning the origin of the electrical responses they have recorded. The subject has been reviewed recently by WULFF (1956). The close proximity of receptor cells and optic ganglion in many compound eyes accounts for some of the difficulty in assigning particular electrical components to one or the other structure. One method of isolating the receptor cell response has entailed surgical removal of the optic ganglion. Responses of the isolated receptor cells have frequently differed from those of the intact preparation. The differences may be explained, it has been argued, by inferring the existence of a ganglionic component in the electroretinogram of the intact preparation. In a strict sense, however, the only type of post-operative receptor cell response which permits a single, simple interpretation is the one which is like that of the intact preparation in all fundamental respects (wave form, amplitude, etc.). All other types of response require consideration of injury effects, a consideration which has not always been taken into account by previous workers.

SVAETICHIN *et al.* (1956) briefly report on another possible source of confusion, namely, that certain of the shielding pigment cells of moth ommatidia respond to illumination with an electrical variation which is superimposed on the receptor cell response.

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Such considerations indicate that it is desirable to study eyes in which photoreceptor cells are naturally well isolated from ganglionic structures and which lack pigment cells whose state alters with level of illumination. The dorsal ocelli of cockroaches satisfy these conditions and are the organs which have been studied in greatest detail in the work reported below. Data from two cockroach species, *Periplaneta americana* and *Blaberus craniifer*, are included in this paper. For comparative interest ocellar responses of the grasshopper, *Melanoplus bivittatus*, are also included. These three species were selected for detailed study following a survey of ocellar responses in seventeen species representing five insect orders. The ocellar responses of all seventeen appear to be based on the same fundamental components, the interaction of which varies from species to species. Some of the material from the remaining fourteen species will be presented at a later time.

Four studies of electrical aspects of ocellar function have appeared in the literature. PARRY (1947) interpreted his data on *Locusta migratoria* to mean that neither illumination nor darkening of the ocellus excited nerve impulses in the ocellar nerve fibres, though he stated in his discussion that additional evidence was needed firmly to establish this point. HOYLE (1955) showed that in *Locusta migratoria* ocellar nerve fibres do, in fact, conduct nerve impulses. These two authors and also RUCK (1954) and BURTT and CATTON (1956) have briefly described the ocellar electroretinogram. None of these contributions, however, gives an adequate account of the origin, polarity, and complexity of the responses. Moreover, they include contradictory data and interpretations. It will be better to return to this in the discussion.

THE STRUCTURE OF THE OCELLUS IN *PERIPLANETA AMERICANA*

The cockroach has two ocelli which appear as white spots on the front of the head. On the average they are larger, hence more convenient for experimental purposes, in males than in females. The cornea is a transparent disk, thicker than the skeleton around it, and approximately flat on both sides. Apparently it functions only as a "window" and not also as a lens.

In Fig. 1, A represents a semi-diagrammatic section in a plane perpendicular to the corneal surface which bisects the ocellus; the left side of the figure is nearest the midline of the head. The figure is based mainly on preparations stained with reduced silver according to the method of SAMUEL (1953), and emphasizes neurological detail. Sections variously fixed and stained with haematoxylin and eosin, Mallory's triple stain, and Heidenhain's azan have provided a clearer picture of other cytological features.

A layer of sense cells occupying about half the thickness of the ocellus lies immediately beneath the cornea. The sense cells occur in groups, the retinulae, of from three to five cells. Specialized structures, the rhabdomeres, occur at the surfaces where adjacent cells come into contact (Fig. 1, C). The rhabdomeres stain grey or black with reduced silver, as do the nerve fibres. Each sense cell has an axon. All the sensory axons of a retinula proceed in a compact group

towards the synaptic region at the base of the ocellus. Axon groups from neighbouring retinulae come together, forming larger groups, in the manner indicated in Fig. 1, A. The diameters of the sensory axons all appear to be less than 1μ.

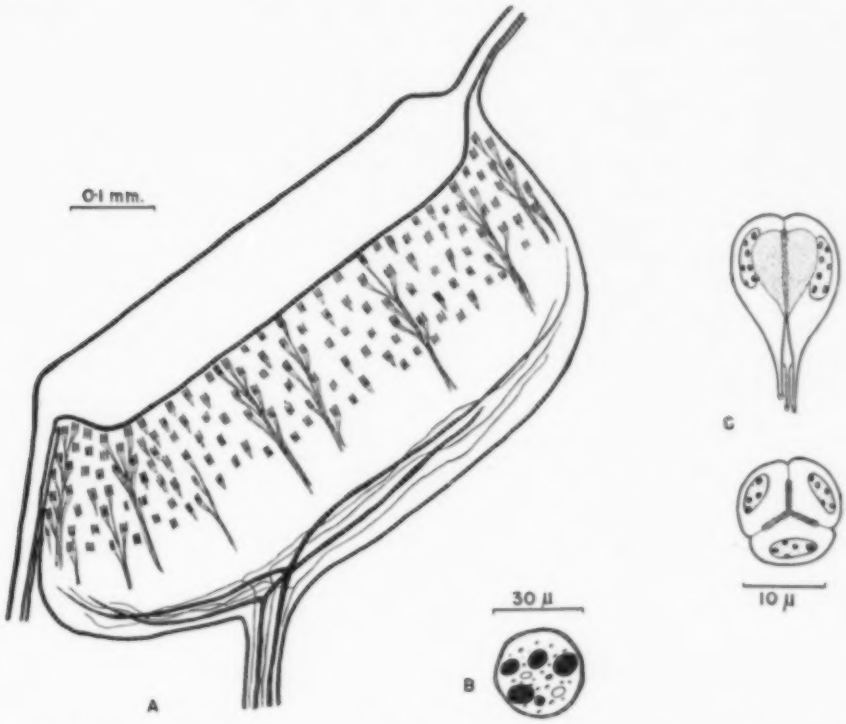

FIG. 1. The ocellus of *Periplaneta americana*. A: a semi-diagrammatic section through the ocellus; receptor cell axons not drawn to full length; see text. B: cross-section of the ocellar nerve; black dots represent ocellar nerve fibres; two tracheae also present. C: detail of a retinula in longitudinal and cross-section; rhabdomeres (stippled) and nuclei within the photoreceptor cells.

The sensory axons leave the sensory layer and appear to form intimate synaptic relationships with the intra-ocellar terminals of the ocellar nerve fibres. Thus far it has been impossible to visualize clearly the nature of the synaptic contacts. Most of the participating units are too fine, their packing too dense. Therefore, in Fig. 1, A the sensory axons have simply not been drawn beyond the clear zone separating the sensory layer and the synaptic region.

The ocellar nerve (Fig. 1, B) appears to contain about twenty-five nerve fibres. There are four large ones with diameters of 6 to 10μ, about twenty small ones (a total arrived at by counting all the minute, silver-stained dots in cross-sections of the nerve) in the 0·5 to 2μ range, and often two fibres of intermediate size.

Their cell bodies have not been found in the ocellus or in the ocellar nerve. CAJAL (1918) tried to locate the cells of origin in the brains of bees and dragonflies but was unable to identify them with certainty. A similar search made in the present study was unsuccessful. While it is impossible at the present time to state where these cell bodies are, it is very unlikely that they are in the ocellus for that small organ has been searched most carefully.

In the zone between the sensory layer and the synaptic region, and surrounding the latter, the roach ocellus is packed with a white material which doubtless functions as a tapetum. This tapetum consists of aggregates of small granules, the larger of which appear as radially striated spheres. They probably consist of urate. There are no shielding pigments in or around the ocellus.

The ocellar nerve on leaving the ocellus proceeds mesad about 0·6 mm, then passes superficially under the sheath of the brain. On reaching the midline it turns posteriorly, joins the nerve from the opposite side, and the two traverse the brain from front to back in the mid-sagittal plane.

EXPERIMENTAL METHODS

Adult male roaches of two species, *Periplaneta americana* and *Blaberus craniifer*, and either males or females of *Melanoplus bivittatus*, were used. In most cases the experimental animal was intact but legs, thorax, and head were rigidly immobilized with tacky wax. All eyes except the experimental ocellus were covered with aluminium foil. The experimental ocellus was positioned in the optical system so that a circular portion of the image of an incandescent strip of tungsten fell on its cornea.

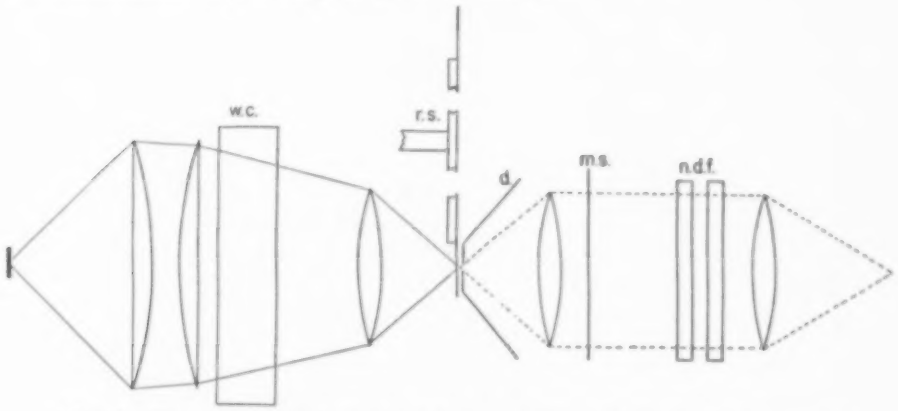

FIG. 2. The optical stimulator. Strip filament tungsten lamp as light source; w.c., water cell; r.s., rotary shutter; d, diaphragm with 2 mm circular aperture; m.s., manual shutter; n.d.f., neutral density filters to control stimulus intensity.

Fig. 2 shows the type of stimulator used. The source was a 6V., 100W., strip filament tungsten lamp. White light was used throughout. The illumination produced at the cornea when no filters were in the path of the stimulating beam ranged from 1,000 to 10,000 foot-candles. This variation arose chiefly because terminal lenses of different focal length were used from time to time to provide different working distances between lens and ocellus. The value, log $I = 0$, refers to the maximum illumination at the cornea

in any one experiment; it may vary from 1,000 to 10,000 foot-candles from experiment to experiment.

Beyond the Wratten filters (n.d.f., Fig. 2) the stimulating beam passed through a hole in the wall of the animal chamber, which otherwise was light-tight and which was painted flat black inside. Experiments were conducted in a dark room.

Except for one case the electrodes used were made from 0·012-in., spring-tempered, stainless-steel wire. Short lengths, electrolytically pointed, were soldered to shielded amplifier leads. An electrode was driven through the ocellar cornea with the aid of a micromanipulator until its tip entered the receptor layer. This passage was observed through a binocular dissecting microscope. The reference electrode was inserted into the flagellum of the antenna of the opposite side. It is worth emphasizing that this site for the reference electrode is the most satisfactory one which has been found in insects with long antennae; it is indifferent to potential variations arising in the ocelli and produces negligible "noise" of its own.

In one series of experiments glass pipette microelectrodes filled with 3M KCl were used. Tip diameters were in the 5 to 10 μ range. Bare metal leads of silver or stainless steel connected the pipette contents with the amplifier input.

It was considered important that wave forms of the receptor potentials be accurately recorded. In all experiments the input circuit included junctions of metal with salt solutions. If such junctions were to polarize to an appreciable extent on application of a potential difference, wave forms would be distorted. But polarization, hence wave-form distortion, decreases as the impedance of the input circuit increases. The amplifier used was the Model P6 of the Grass Instrument Co. The input stage of this amplifier is a cathode follower circuit with a very high impedance. It was directly coupled to the electrodes and was operated push-pull. Polarization effects were unnoticeable in the experiments. The amplifier output was led into the differential input of a dual-beam oscilloscope.

The following convention will be used in all the text-figures: negativity of the ocellar electrode gives an upward deflection of the oscilloscope sweep.

Additional details concerning methods appear in the text.

RESULTS

1. *Wave form of the ocellar response as a function of log I and state of adaptation*

All the data described in this section were obtained using stainless-steel electrodes, one in the receptor layer, the other in an antenna. The dark adapted (DA) ocellus is one which has not been exposed to light for at least 2 min. It was found that 2 min were sufficient for ocellar electrical responses to recover almost completely after a 5 min exposure to a light producing a corneal illumination of 10,000 foot-candles. The effects of prior light adaptation on wave form and amplitude of responses taken after 2 min in the dark are negligible for present purposes. (Details of the dark adaptation experiments will be reported elsewhere.) The light adapted (LA) ocellus, arbitrarily defined, is one which has been stimulated at a rate of one flash per second, with a flash duration of $\frac{1}{8}$ sec, until the wave form of the response has stabilized. At log $I = 0$ repetitive stimulation for as long as $1\frac{1}{2}$ min was often required for stabilization.

In Fig. 3, Plate 1, responses of the *Periplaneta* ocellus to $\frac{1}{8}$ sec. flashes are shown for the DA and the LA states from log $I = -7{\cdot}0$ to log $I = 0$. Stimulus intensity increases from top to bottom. Amplification is not the same for all the responses.

The lowest stimulus intensity which produces a visible response in *Periplaneta* elicits an off-effect in the form of a rapid (duration of 2 to 4 msec), positive deflection. This is clearly visible at log $I = -7{\cdot}0$, LA. As stimulus intensity increases this off-effect increases in amplitude in an abrupt, stepwise manner. The greatest amplitude has been attained at log $I = -4{\cdot}0$. Never have more than four increments of growth been observed; most often there are only three. Sometimes this off-effect takes the form of two, three, or four separate, rapid, positive deflections. Infrequently, in the same ocellus, one stimulus elicits one large off-effect, the next, two or three smaller ones. It is likely that this activity arises in the largest ocellar nerve fibres, that they are recruited with increase of intensity in the low intensity range, and that they most often fire once per fibre and synchronously. The units of this off-effect will be called "off-spikes". There is always a level of stimulus intensity above which they no longer appear; in Fig. 3 it corresponds to log $I = -2{\cdot}0$.

The threshold for the off-spikes may be the same as for a positive deflection at "on", but more often the positive on-effect appears at a slightly higher intensity (Fig. 3, log $I = -6{\cdot}0$). With further increase of intensity (log $I = -5{\cdot}0$) the positive on-effect is followed by a negative wave. At all intensities higher than log $I = -6{\cdot}0$, the response contains both positive and negative components. At log $I = -2{\cdot}0$, and at higher intensities, the negative component has the shorter latency at "on" in the DA response.

At log $I = -4{\cdot}0$, DA, there are two points of inflection on the ascending limb of the negative potential. At log $I = -3{\cdot}0$, DA, and at higher intensities, there is a single, conspicuous notch in the same region, and the first positive wave has become a much more rapid deflection. At some intensities repetitive stimulation can produce this sequence of variations at "on" in a very pronounced manner, as at log $I = -2{\cdot}0$, LA. These responses convey the impression that under some conditions of stimulation a positive and a negative component interact so as to produce a resultant oscillation, which in *Periplaneta* is highly damped. Such oscillations, in greater or lesser degree, are characteristic of many of the insect species studied, but nowhere have they appeared so prominently as in the honeybee ocellus (RUCK, 1954).

The highest stimulus intensities produced in the LA ocellus a marked decline in amplitude of the negative slow wave, and an irregular sequence of variations at "on" (Fig. 3, log $I = -1{\cdot}0$ and 0, LA).

In the grasshopper, *Melanoplus bivittatus*, the positive and negative components of the ocellar response were most distinctly developed. The responses of *Melanoplus* in Fig. 4 were recorded under the same conditions as those of *Periplaneta* in Fig. 3. Amplification is the same throughout Fig. 4. The threshold response in *Melanoplus* is a positive slow wave. At intensities higher than log $I = -5{\cdot}0$ the negative component enters, becoming more prominent in the DA ocellus up to log $I = -1{\cdot}0$. Off-spikes are visible only at log $I = -5{\cdot}0$ and $-4{\cdot}0$. At log $I = -3{\cdot}0$ and higher intensities, light adaptation is accompanied by a proportionately greater decrease of the negative component.

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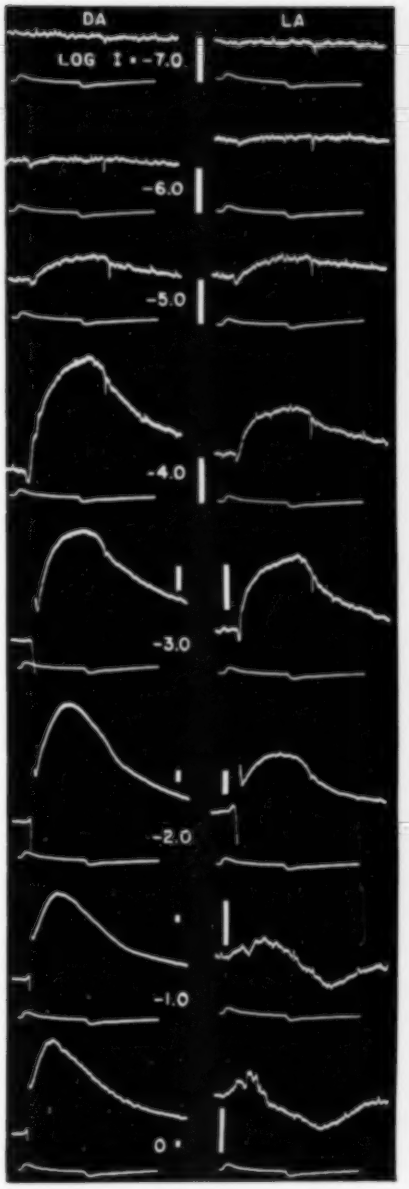

FIG. 3.

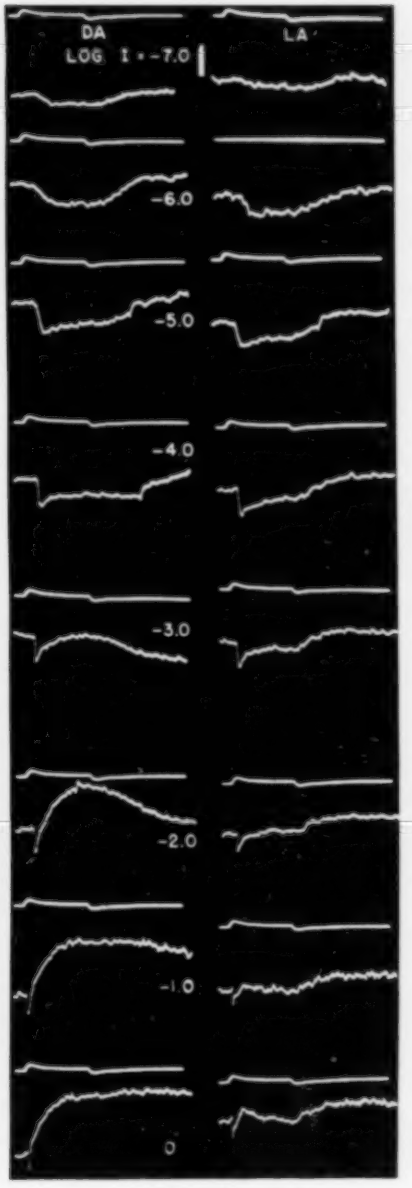

FIG. 4.

PLATE 1

FIG. 3. *Periplaneta*. Ocellar responses of normal, intact animal in upper trace. One electrode in receptor cell layer, the other in the antenna of the opposite side. Stimulus signal marker in lower trace; stimulus duration = $\frac{1}{4}$ second. Height of all calibration marks (vertical white strips) = 1 mV; when calibration is given between DA and LA responses it applies to both.

FIG. 4. *Melanoplus*. Responses of median ocellus of normal, intact animal in lower trace. Calibration between uppermost responses = 1 mV and applies to all responses. Conditions otherwise the same as in Fig. 3.

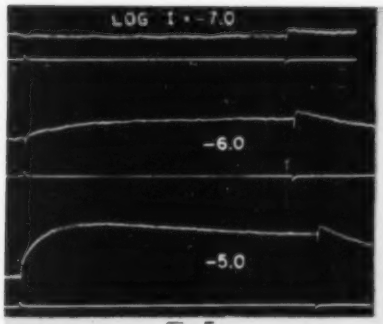

Fig. 5

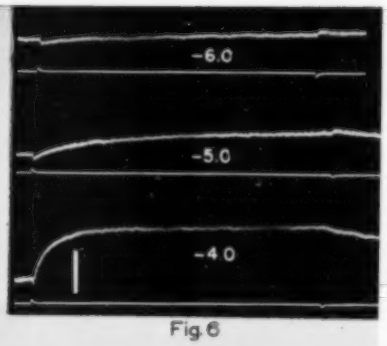

Fig. 6

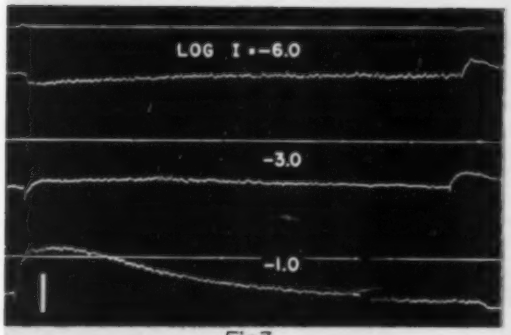

Fig. 7

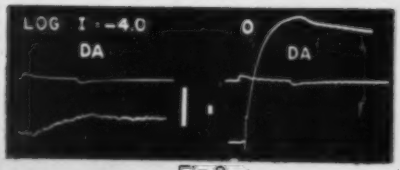

Fig. 8

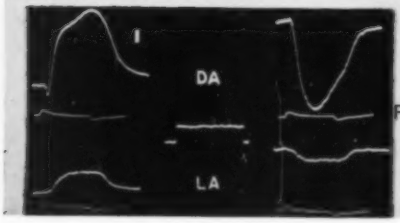

Fig. 9

Fig. 10

PLATE 2

FIG. 5. *Periplaneta.* Responses of ocellus in normal, intact animal. Stimulus duration about 5 seconds. Calibration = 1 mV. Conditions otherwise as in Fig. 3.

FIG. 6. *Periplaneta.* Responses of ocellus recorded about 5 minutes after cutting the ocellar nerve. One electrode subcorneal in receptor layer, the other in the antenna of the opposite side. Log *I* as indicated. Calibration = 1 mV. See text.

FIG. 7. *Melanoplus.* Responses of median ocellus of normal, intact animal. Stimulus duration about 5 seconds; signal marker failed at log *I* = −3·0 and −1·0. Calibration = 1 mV. Conditions otherwise as in Figs. 3 and 4.

FIG. 8. *Periplaneta.* Responses of an ocellus the nerve of which had been severed 8 days previously. One electrode subcorneal in receptor layer, the other in the antenna of the opposite side. Stimulus duration = $\frac{1}{4}$ second. Both calibration marks = 1 mV. See text.

FIG. 9. *Periplaneta.* Response of an ocellus wholly isolated on an agar plate. Responses at the left taken with one electrode at surface of receptor layer, the other several centimetres away in the agar; at right, the active electrode was on the back of the ocellus. Stimulus duration = $\frac{1}{4}$ second. Calibration = 1 mV and applies to all records. See text.

FIG. 10. *Blaberus.* LA ocellar responses of normal, intact animal to $\frac{1}{4}$-second flashes. Records 1 (surface of receptor cell layer) through 6 (back of ocellus) taken at successively greater depths with a glass micropipette electrode. Indifferent electrode in antenna of opposite side. Calibration = 1 mV and applies to all records. See text.

Certain additional features appear both in *Periplaneta* and *Melanoplus* with stimulus durations greater than $\frac{1}{8}$ sec. In Fig. 5, Plate 2, appear responses of *Periplaneta* at lower intensities to stimuli of about 5 sec duration. At log $I = -7{\cdot}0$ there is a very low amplitude positive deflection at "on" and no other deviation from the baseline until "off". Then, in order, appear the off-spikes and a slow negative wave which did not appear with $\frac{1}{8}$-sec stimuli. Except that a negative wave dominates while the light is on, the responses at log $I = -6{\cdot}0$ and $-5{\cdot}0$ show the same features.

Fig. 7 shows the responses of *Melanoplus* to 5 sec exposures. At log $I = -6{\cdot}0$ the positive wave is dominant during illumination; at "off" there is a negative rise as in *Periplaneta*. At log $I = -1{\cdot}0$ a large negative wave rises to a maximum while the light is on; both the off-spikes and the slow negative off-wave are absent at this intensity. The corresponding response of *Periplaneta* at high intensity is almost identical, though not shown.

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2. *The effect of severing the ocellar nerve in Periplaneta*

The responses of the normal ocellus consist of several components. The simplest interpretation seems to be that there are three basic ones, a positive and a negative slow wave, which algebraically sum and which may interact in an oscillatory manner, and off-spikes. Certain evidence presented in the last section is consistent with the view that the off-spikes arise in the four largest ocellar nerve fibres, and supporting evidence will be presented below. Though it may appear probable that the ocellar electroretinogram, minus the off-spikes, originates in the photoreceptor cells it is best to consider all possibilities. Taking the most inclusive view, portions of the electroretinogram may arise in (1) the photoreceptor cells, (2) the intra-ocellar terminals of the ocellar nerve fibres, and (3) the brain.

Any part of the total response which remains after severing the ocellar nerve cannot have originated in the brain. After ocellar nerve section (see below) every component of the normal response, except off-spikes, remained (Figs. 6 and 8).

Available histological evidence indicates that cell bodies of the ocellar nerve fibres lie in the brain. In this case, cutting an ocellar nerve between brain and ocellus may be expected to result in degeneration of the intra-ocellar terminals, and any component of the electrical response remaining a week or so after nerve section is likely to be of photoreceptor cell origin. Ocellar nerves were severed in a large number of roaches (about sixty) and the responses of the ocelli were studied from 2 to 8 days after the operations. These post-operative responses contained all the components described for the intact ocellus, but in all cases wave forms and thresholds differed somewhat from the normal. The operated ocelli were not studied histologically to determine whether or not the intra-ocellar terminals of the nerve fibres had in fact degenerated.

In one type of operation a flap of cuticle was raised from the front of the head and sufficient tracheae and glandular tissue were removed so that the brain could be seen clearly. The blades of a fine iridectomy scissors were placed

with their tips above and below the point where one of the ocellar nerves entered the brain. A single cut was made partway into the brain. The skeletal flap was folded back and waxed in place. Such animals were studied 2 to 8 days later.

In Fig. 8 are shown the responses of an ocellus the nerve of which had been sectioned 8 days previously. In response to $\frac{1}{8}$ sec flashes the positive and negative components are still present. But the threshold of this ocellus was higher than normal, and the threshold response was a negative wave rising at "on" and falling at "off". At higher intensities the positive component entered, and at the highest intensities the response appeared quite normal with respect to wave form and amplitude. In about sixty animals studied in this way the positive component was feebly developed at low stimulus intensities. The slow negative off-wave which appears in normal ocelli with 5 sec exposures (Fig. 5) never appeared in these operated ocelli. The simplest interpretation of that wave is that it represents the algebraic sum of a positive component, which returns to the baseline rapidly at "off", with a negative wave which falls slowly; in the operated animals the positive component, weakly developed at low intensities, cannot contribute to events at "off". The off-spikes never appeared after ocellar nerve section. What has been said applies equally to animals studied from the second to the eighth day after nerve section.

One ocellar nerve was left intact in the above animals. The ocellus associated therewith was studied as a control in a number of cases and was often found capable of responding in an apparently normal manner. Therefore, it can be concluded that the last phase of the operation, that in which the ocellar nerve of the opposite side was cut, was responsible for the alterations of response on the cut side.

Experiments were conducted to determine the length of time required after cutting the nerve for the off-spikes to disappear and for deterioration of the positive component to set in. It became apparent that this time is very short, a matter of a few minutes. Animals were placed in the optical system and not moved again during the operations. The top of the head was sliced away, the ocellar nerves and brain exposed. Electrodes were placed (subcorneal in ocellus *v.* antenna) and the response was established to be normal before severing an ocellar nerve. The ocellar electrode was withdrawn, the ocellar nerve cut; the electrode was replaced and the preparation was allowed 2 min to dark-adapt. About 4 min. elapsed during and after the operations before the ocellar responses could again be studied.

Fig. 6 shows the responses of an ocellus treated in the way just described. The record at $\log I = -6{\cdot}0$ was obtained 5 min after the normal pre-operational control response. The off-spikes are absent from this ocellus. They have never appeared after cutting the nerves, nor have they appeared when the ocellar nerve fibre tract was cut near the posterior surface of the brain without so much as touching the nerves. But the other response components are present in Fig. 6. The negative off-wave is, however, more feebly developed than in the normal ocellus (Fig. 5). Fifteen minutes after operating on the animal the

electrode was shifted to the ocellus with the intact nerve. Its responses were essentially normal in all respects.

A likely interpretation of these results is: (1) That differences of ocelli with severed nerves from normal ocelli do not depend on degeneration of ocellar nerve fibres. They occur too rapidly. The act of cutting the ocellar nerve (tracheae, etc.) on one side of the head affects only the ocellus of that side. (2) No basic component of the ocellar response, except off-spikes, is removed by nerve section. (3) It is likely that the peripheral portions of the ocellar nerve fibres degenerate by the eighth day, yet all components of the ocellar electrical response, except off-spikes, are still present on the eighth day. Therefore it is likely that the ocellar electroretinogram, except for the off-spikes, originates in the photoreceptor cells.

3. *Polarity of the ocellar response as a function of electrode position*

Responses of roach ocelli, the soft tissues of which had been totally isolated from the head, were recorded from the surface of the receptor layer and from the surface closest to the brain. Agar gels made up in insect saline (HOYLE, 1953) were prepared. A roach head was opened, an ocellar nerve was cut, and a piece of cuticle including the ocellar cornea, to which the soft tissues of the ocellus adhered, was removed from the head. The back of the ocellus was lowered on to the agar plate and the receptor layer was freed from the cornea. The cornea was discarded and the preparation consisted of an isolated ocellus, the back of which was applied to the surface of the agar. A blunt, stainless steel-electrode was placed on the freely exposed surface of the receptor layer and another was thrust into the agar plate several centimetres distant.

The ocellus was stimulated with $\frac{1}{8}$ sec flashes at $\log I = 0$ and the responses at the left of Fig. 9 were recorded. The DA response contains the positive and negative components previously associated with the normal ocellus, but rates of rise and fall of the positive component are slower than in the normal preparation. In the LA response this component scarcely appears. In only two cases out of thirty did it appear, and in these it survived only for a few minutes. The negative component, however, was present and strongly developed in all thirty, and survived for at least half an hour even when the preparation was simply allowed to dry up. The positive component is therefore very labile, while the negative one is very resistant to adverse treatment.

After recording in the above manner the ocellus was turned over so that the surface of the receptor layer faced the agar. The active electrode was placed on the back of the ocellus, and the responses at the right of Fig. 9 were recorded. Whereas in the previous case the dominant response was a large negative wave it is now a large positive one, and the labile component has almost disappeared. It too, however, has inverted so that it appears negative to the electrode on the back of the ocellus (DA response).

Obviously polarity depends on the location of the recording electrodes. Potentials which appear positive to an electrode at the surface of the

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receptor layer appear negative to one at the rear surface of the ocellus, and vice versa.

Assume for the moment that the component with negative sign at the surface of the receptor layer depends on the flow of electrical current from extra-cellular spaces into the distal, expanded ends of the sense cells (Fig. 1). The potential of the extra-cellular space in this region will have a negative sign. But all the current which flows into the cells in one region must flow out of them elsewhere. In regions where the flow from cells to extra-cellular spaces exceeds the flow in the reverse direction, the potential sign of the extra-cellular spaces will be positive. If current flows inward only at the distal ends of the receptor cells it must flow predominantly outward from the sense cell axons to the medium. The same argument with reversed signs applies to the component whose sign at the surface of the receptor layer is positive. This general argument has been well developed in different contexts (*e.g.* LORENTE DE NÓ, 1947; HOWLAND *et al.*, 1955).

If an electrode capable of recording the potential of a very small volume of ocellar space were advanced from the surface of the receptor layer through the ocellus, the potential of either response component, referred to an indifferent electrode, should reach zero at some depth, then reverse beyond. On reaching the zero point one would know that the "active" sites lie either backward or forward along the electrode path.

Potential as a function of depth was recorded in the ocellus of *Blaberus craniifer*. The ocelli of this animal have the same anatomy as in *Periplaneta*, but are much larger organs. The cornea has twice the diameter, and the depth from front to back is almost twice as great. The responses of the *Blaberus* ocellus had been previously found to be almost identical under all conditions of stimulation to those of *Periplaneta*. As a preliminary to the experiments, an ocellus of one *Blaberus* was sliced in half by a cut perpendicular to the cornea which passed through the centre of the cornea. This ocellus was used to "calibrate" the travel of an Emerson mechanical micromanipulator. The manipulator was adjusted so that the maximum movement of the electrode carriage corresponded to the distance from the surface of the receptor layer to the back of the ocellus, a distance of about 0·55 mm.

Having determined this distance, an intact *Blaberus* was mounted and a small hole made in the centre of the cornea. A glass pipette microelectrode was fixed to the electrode carriage. The tip was advanced into the hole in the cornea until it lay approximately at the surface of the receptor layer. Movement of the manipulator control lever through its full traverse would then advance the electrode tip 0·55 mm, *i.e.* approximately to the back surface of the ocellus. The indifferent electrode was placed in the antenna of the opposite side.

The ocellus of Fig. 10, Plate 2, was stimulated at $\log I = -2{\cdot}0$ with $\frac{1}{4}$ sec flashes until the equilibrium (LA) response appeared. A recording was made at the surface of the receptor layer; this is response number 1. The electrode tip was advanced and records 2 through 6 were obtained at successively greater depths. Response number 3 was recorded about half-way through the ocellus,

or approximately at the junction of the layer of receptor cell bodies and the layer of sensory axons. Number 4 appeared very slightly deeper. The early part of response number 4 (to the left of the number, 4) has inverted; the latter part of the response (right of the number, 4) has not inverted. In response number 5, recorded in or very near the synaptic region, the electroretinogram, except for the off-spikes, has wholly inverted. Clearly, portions of the electroretinogram taken at different times during the responses may pass through zero points at different depths in the ocellus. This may indicate that during a response the active sites expand, so to speak, to include greater lengths of the receptor cells.

Response number 6 was recorded approximately at the rear surface of the ocellus, *i.e.* at maximum traverse of the micromanipulator, and amplitudes of all components had decreased. No new components appeared while passing through the synaptic region. The polarity of the off-spikes remained unchanged from 1 through 6.

Penetration and withdrawal could be repeated on the same ocellus. Similar results were obtained in six experiments on three animals.

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The significance of these data can only be: (1) That all components of the ocellar response, except the off-spikes, originate in the photoreceptor cells and distal to the region where polarities reverse, namely, in the region where the rhabdomeres and cell bodies are concentrated. The greater magnitude of the potentials as seen at or near the surface of the receptor cell layer probably depends on the fact that the area of active receptor cell surfaces is smaller than the area of passive receptor cell surfaces which complete the "circuit". This condition would make current flow more dense, potentials therefore greater, in the region of the active sites. (2) Slow potentials originating in the synaptic region, hence in the terminals of the ocellar nerve fibres, are not resolvable with this or any of the methods which have been used.

Whether all components of the electroretinogram which originate in the photoreceptor cells arise at the same, or different, sites cannot be determined from the data thus far collected. If it could be shown that the polarity of each component reverses at a different depth it would favour the view that each arises at a different site. Experiments designed to resolve this problem will depend on use of a micromanipulator permitting very small, straight-line movements of precisely measured extent.

4. *The origin of the off-spikes*

Attempts to record the activity of the ocellar nerve fibres by placing electrodes directly on the ocellar nerves have failed so far. The evidence that the off-spikes originate in the large ocellar nerve fibres is therefore indirect. An electrode in an ocellus records the off-spikes only when the ocellar nerve is intact. If the ocellar nerve fibres are sectioned anywhere along their course the off-spikes are gone as soon as the preparation can be tested. The possibility exists that the off-spikes are confined to the brain and "seen at a distance" by the ocellar electrode, but this can be tested.

If the off-spikes appear at the ocellus because the ocellar nerves somehow act passively as electrical leads from the brain, stimulation of one ocellus ought to cause off-spikes to appear at the other ocellus because the ocellar nerve fibres form a common tract, a single lead, almost to the point where they leave the brain. Electrodes were placed in both ocelli of *Blaberus* and were referred to a common antennal electrode. The cornea of one ocellus was shielded from all light incident on the other cornea. Thresholds for the off-spikes were determined for both ocelli while directly illuminating only one. In the two cases studied the off-spike threshold was about a thousand-fold lower for the ocellus directly exposed to the stimulus. The shielded ocellus was undoubtedly stimulated by light passing through the tissues of the head. This result precludes the possibility that the off-spikes never leave the brain, and demonstrates that off-spikes appearing in one ocellus are elicited only by that ocellus.

The polarity of the off-spikes now deserves closer attention. In Fig. 10, Plate 2, it can be seen that the initial phase of the off-spikes is a rapid positive deflection; this is followed by a rapid negative overshoot which declines relatively slowly. This sequence of polarity changes is exactly what one would expect to record in a volume conductor as the sign of nerve impulses propagating from the brain towards the ocellus. As the impulses approach, the ocellus acts as a current "source", when they arrive as a "sink".

This interpretation fits well with the observation that the off-spikes disappear immediately on sectioning the ocellar nerve fibres. From this it must be concluded that the off-spikes are efferent impulses in the large ocellar nerve fibres. Therefore, the electrical responses so far recorded provide no evidence of the afferent impulses which must originate in the ocellus in order to initiate the efferent off-volley.

DISCUSSION

Evidence has been presented which indicates that electrical responses of photoreceptor cells in roach and grasshopper ocelli contain at least two components, that these are of opposite electrical sign, that they sum algebraically and may interact to produce oscillations. The strongest evidence for their origin in the receptor cells was described in section 3, and is briefly summarized as follows. Each component had one polarity when recorded at the surface of the receptor layer, the opposite polarity when recorded from the back surface of the ocellus. A microelectrode passed through the ocellus encountered a region where the polarities of both components reversed. This region, lying between the layer of receptor cell bodies and the synaptic region, corresponds to the layer of sensory axons. An interpretation based on the flow of current in a volume conductor led to the conclusion that both components originated in the layer of receptor cell bodies.

Yet the same types of experiments which yielded these data in the roaches have yielded quite different data in *Locusta migratoria*. BURTT and CATTON (1956) state that they passed a microelectrode through the locust ocellus from front to back and did not observe polarity reversal. (The polarity of the response they recorded was not specified.) For physical reasons (see section 3) it would seem

that there must be a polarity reversal along some ocellar axis. There are, however, a large number of lines which can be passed through an ocellus along which polarity reversal would not be expected to occur (e.g. any line wholly confined to the layer of receptor cell bodies); it seems possible that their electrode path, or paths, may have lain along one or more of such lines.

BURTT and CATTON (1956) also passed microelectrodes through the ommatidial layer and into the optic ganglion of the compound eye in four insect species. They found that in all cases the polarity of the electroretinogram reversed in a sharply localized region corresponding to the first synaptic layer, the *lamina ganglionaris*. They concluded that the region of polarity reversal was the site of origin of the electroretinogram, but did not make clear the physical assumptions on which their conclusion was based. An interpretation of the same data, consistent with the distribution of current in a volume conductor surrounding elongated, parallel nerve cells, would forbid the conclusion that the region of reversal is the site of origin of the electroretinogram. Rather, the site of origin would lie backward or forward along the path of penetration of the microelectrode, specifically, in the ommatidia or in portions of the optic ganglion central to the *lamina ganglionaris*. When they removed the optic ganglion central to the *lamina* and found that the electroretinogram persisted, they in fact localized the site of its origin to the ommatidia.

PARRY (1947) and HOYLE (1955) also recorded the ocellar electroretinogram of *Locusta migratoria*. With two electrodes on the ocellar nerve, and the preparation immersed in the recording medium, the dominant slow potential that both recorded was similar. The electrode nearest the ocellus became positive during illumination of the ocellus; on darkening, the positive wave declined and the response ended in a small negative overshoot.

None of the roach or grasshopper responses described in this study have been recorded in exactly the same manner. However, it was found that with one electrode on the back of an isolated roach ocellus, the other at a remote point in the recording medium, the dominant slow potential was positive in sign. Responses of ocelli *in situ* but wholly exposed to the air have been recorded (but not previously described) in *Melanoplus bivittatus*, *Dissosteira carolina* (Carolina grasshopper), and *Libellula vibrans* (dragonfly). One electrode was placed on the back of the ocellus, the other at a remote point in the body (leg or abdomen). In these species the dominant slow component of the response, with high intensity stimulation (1,000 foot-candles at the cornea), was positive in sign. (The same component appeared negative when the electrode was placed at the surface of the receptor layer.) Therefore, if only the one recording situation of PARRY and HOYLE, noted above, is considered our results do not disagree with respect to this one wave.

With other positions of the recording electrodes the polarity of the responses recorded by PARRY and HOYLE do not agree, and cannot be compared with the data of this study. Their recording situations, e.g. any tissue of the head *v.* ocellar nerve (PARRY), and the partly immersed ocellus *in situ* *v.* saline pool near ocellus (HOYLE), have not been used in this study. Neither PARRY nor HOYLE recorded

both from the surface of the receptor layer and from the back of the ocellus, nor did either record from wholly isolated ocelli. Such recordings might resolve their present disagreement.

Comparison of ocellar responses of *Locusta* with those of the roaches and grasshoppers in this study is rendered somewhat difficult because stimulus intensities were not specified for any of the *Locusta* data. One of the most important determinants of the wave form of ocellar electrical responses is the strength of stimulation (Plate 1).

HOYLE (1955), with two electrodes on the ocellar nerve of *Locusta*, also recorded smaller, shorter duration responses to illumination and darkening which he identified as nerve impulses in ocellar nerve fibres. One large, rapid wave, negative in sign at the electrode nearest the ocellus appeared at "on". HOYLE believed that this wave represented nerve impulses in the two largest of the six ocellar nerve fibres present in *Locusta*. The possibility exists, however, that this on-wave actually originated in the photoreceptor cells of *Locusta*, and is equivalent to that part of the high intensity responses in *Periplaneta* and *Melanoplus* (Plate 1), which appears as a rapid positive deflection at "on" when recorded at the surface of the receptor layer. RUCK (1954) described an analogous component (as "spikes") in records from the honeybee ocellus. Now it is known that this wave, which is also present in roaches, several grasshoppers, including *Melanoplus*, and all the other species which have been studied, is produced through interaction of a positive and a negative component of receptor cell origin. In flickering light at higher intensities this on-wave may follow each flash at lower flicker rates, frequently fail at intermediate rates, and finally fail altogether at some higher flicker rate. This sequence may be seen in the honeybee records of RUCK (1954). HOYLE's statement that the rapid on-wave of *Locusta* behaves in the same manner in flickering light increases the likelihood that it originates in the photoreceptor cells of *Locusta*.

In addition to the above component, HOYLE recorded smaller, shorter duration waves which probably represented propagated impulses in ocellar nerve fibres. The nerve was spontaneously active in the dark; illumination inhibited the activity. At low levels of illumination, in which the nerve was silent, HOYLE reported that a sudden decrease of illumination elicited "single firing in one or two axons". It seems possible that these impulses originated in the two largest ocellar nerve fibres. The conditions of stimulation which elicited them in *Locusta* correspond to those which elicit the off-spikes in roaches.

The only interpretation of the off-spikes consistent with the data from *Periplaneta* and *Blaberus* is that they are efferent impulses in the four largest ocellar nerve fibres. It is a curious fact that the sudden appearance of these impulses is the usual indication that threshold has been reached. This means that the threshold response of the photoreceptor cells has not yet been identified.

Concerning the nature of the threshold afferent signal in the ocellar nerve fibres there is, perhaps, some pertinent information, though it comes from *Locusta* and not from the roaches. HOYLE (1955), as stated above, found that ocellar nerve

fibres (probably the smallest ones) were spontaneously active in the dark, and that illumination inhibited this activity. The resumption of firing in small afferent fibres on darkening the ocellus might act as the effective "trigger" for the off-spikes at a more central synapse.

No decisive evidence concerning the rôle efferent impulses may play in ocellar function exists at the present time.

The ocelli of *Periplaneta*, *Blaberus*, and *Melanoplus* are highly sensitive photoreceptor organs. The convergence of many photoreceptor cells on few ocellar nerve fibres, and the tapetum, predispose one to this view. On sudden darkening, some *Periplaneta* ocelli have been found to respond with at least one off-spike when the prior level of illumination lay between 10^{-4} and 10^{-5} foot-candles. It would be a valuable contribution to determine the absolute threshold at the wave-length of light to which the ocelli are most sensitive.

One problem which this investigation has raised concerns the complexity of the photoreceptor cell response and the apparent homogeneity of the receptor cell layer. All of the receptor cells which could be resolved in the histological preparations appeared to be of the one type shown in Fig. 1, C. CAJAL (1918) gave no indication that it was otherwise in the ocelli he studied. It is quite possible that cells which look alike produce different types of electrical response under the same conditions of stimulation, but it is also possible that each cell contributes something to the positive and to the negative "massed" responses which have been recorded.

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ÜBER BELICHTUNGSPOTENTIALE IN DEN AUGEN DER FLIEGEN *SARCOPHAGA* UND *ERISTALIS*

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Abstract—Illumination potentials have been recorded in the eyes of flies by coarse steel electrodes and d.c. amplification. Three types of light stimuli have been used: simple illumination, irregular illumination, and electronic flash. One to 3½ hours after the preparation of the head of the experimental animal, the on- and off-effects of the ERG (in *Sarcophaga*) diminish rapidly and disappear without affecting the negative plateau potential which keeps the same height as before (Figs. 2, 3). The time course of the on-effect and of the isolated negative potential (the latter if stimulated by a flash) may follow an exponential law. The time constants are much shorter in the negative potential than in the on-effect (Figs. 1b, 6b). The on- and off-effects in co-operation form the differential quotient of the time course of changing illumination (Fig. 4).

1. VERSUCHSTIERE UND METHODIK

Die Fliegen wurden im Freien gefangen. Mit einer Rasierklinge wurden Kopf und vordere Thoraxhälfte vom übrigen Körper abgetrennt. Eine der Elektroden wurde von hinten durch den Thorax bis in den Kopf hinein geführt und damit gleichzeitig das Präparat fixiert. Die andere Elektrode wurde von außen her in die Mitte eines der Augen rund 1 mm weit eingestochen. Verwendet wurden Stahlelektroden, d.h. normale dünne Insektennadeln. Durch die großen Kontaktflächen zwischen den Metallelektroden und dem Gewebe wurde reine Gleichstromableitung ermöglicht. Daß dies gelang, geht einerseits aus den Abbildungen 1 bis 3 hervor, entspricht andererseits den Untersuchungen Burkhardts über das Verhalten verschiedenartiger Elektroden bei variierenden Frequenzverhältnissen (mündliche Mitteilung über unveröffentlichte Arbeiten). Die Elektroden wurden durch kurze Verbindungen an den Batterieverstärker Philips GM 4570 angeschlossen, der auf Gleichstromverstärkung geschaltet wurde. Der Eingangswiderstand des Verstärkers ist 2,2 Megohm. Der Ausgang dieses Verstärkers wurde mit dem Gleichspannungsverstärker GM 4530 verbunden, wobei eine zwischengeschaltete Anodenbatterie mit Potentiometer den Gleichstromanteil des Ausgangs des Vorverstärkers kompensierte. Der Ausgang des Gleichspannungsverstärkers wurde direkt an die Platten eines Elektronenstrahloscillographen geführt. Als Registrierkamera wurde Typ FE 106 (Voigtländer-Philips) verwendet.

2. DAS ERG AUF HELL- UND DUNKELREIZE

Das Präparat wurde mit Hilfe einer Projektionslinse von einer 3,5 V Taschenlampenbirne beleuchtet. In den Strahlengang war ein Compur-Kameraverschluß

eingefügt. Die elektrischen Belichtungspotentiale der Versuchstiere auf einen ca. 1 sec dauernden Lichtreiz zeigen Abb. 1(a), 2(a) und 3(a). Das ERG hat die von AUTRUM beschriebene Form: Es umfaßt einen positiven Ein-Effekt, einen negativen Aus-Effekt, sowie ein von AUTRUM so genanntes „ negatives Zwischenpotential "; dieses ist in Abb. 2(a) verhältnismaßig stärker ausgeprägt als in Abb. 1(a) und 3(a), vermutlich infolge verschiedener Position der Elektroden.

Ausmessung der Abklingkurven von Ein- und Aus-Effekten

Der Form einer Abklingkurve kann man u.U. ansehen, um was für einen dynamischen Zusammenhang es sich bei dem abklingenden Vorgang handelt. Die Abklingkurve, die dem einfachsten möglichen Grundvorgang gehorcht, nämlich der Entleerung eines beliebigen Energiespeichers ohne Gegenwirkung, ist die Exponentialfunktion. Die einfachste Methode, um den exponentiellen Verlauf einer Abklingkurve nachzuprüfen, ist die Übertragung der gemessenen Kurve in einen halblogarithmischen Maßstab. Eine exponentielle Abklingkurve wird in diesem Maßstab zu einer Geraden. Eine hyperbolische Abklingkurve behält bei dieser Übertragung ihre Krümmung teilweise bei; sie wird zu einer Geraden bei Übertragung auf doppelt logarithmisches Millimeterpapier.

In Abb. 1(b) sind Ein- und Aus-Effekt von Abb. 1(a) in halblogarithmischen Maßstab übertragen worden. Die Verbindung zwischen den Meßpunkten erweist sich beim Ein-Effekt mit guter Annäherung als eine Gerade; die Kurve des Aus-Effektes bleibt teilweise gekrümmt (man sieht es dem Aus-Effekt auch auf Abb. 1(a) an, daß er zunächst schneller und dann langsamer abklingt als der Ein-Effekt). Die Zeitkonstante einer Exponential-Abklingfunktion ist definiert als diejenige Zeitdauer, in welcher die Funktion auf $1/e$ ihres Wertes, d.h. auf 36,8% ihrer Amplitude absinkt. Die Zeitkonstante des auf Abb. 1(b) gezeigten Ein-Effektes ist 350 msec. Die Trägheiten der Ein- und Aus-Effekte bei *Sarcophaga* (Abb. 2(a), 3(a)) liegen in der gleichen Größenordnung. Die Krümmung der Kurven ist weniger gleichmäßig als auf Abb. 1(a).

Das Verschwinden der Ein- und Aus-Effekte

Bei *Sarcophaga* zeigte es sich in sämtlichen Experimenten, daß sich das gemessene ERG nach 1 bis 3,5 Std. ohne erkennbaren äußeren Einfluß grundlegend verwandelte: Aus dem sog. diphasischen Zustand mit Ein- und Aus-Effekten ging es in einen monophasischen Zustand über. In diesem Zustand brachte ein Lichtreiz eine rein negative Potentialausleitung hervor (Abb. 2b, 3b). Eine solche Umwandlung des ERG von dem diphasischen in den monophasischen Zustand ist von AUTRUM und Mitarbeitern bei *Calliphora* gelegentlich als kurzes Übergangsstadium beim Absterben eines Präparates beobachtet worden (mündliche Mitteilung). AUTRUM und GALLWITZ (1951) beschrieben das Negativwerden des ERG von *Calliphora* als Folge präparativer Entfernung des 2. und 3. optischen Ganglions von *Calliphora*. Es handelt sich bei der beobachteten Umwandlung also wahrscheinlich um ein lokal begrenztes Absterben bestimmter Gewebe bzw. Funktionen. Das regelmäßige Auftreten der Umwandlung vom diphasischen zum

monophasischen Zustand ohne jede vom Experimentator gesetzte Änderung in der Versuchsanordnung erlaubte es bei *Sarcophaga* und *Eristalis* (bei welcher das gleiche eintrat), die quantitativen Verhältnisse zwischen dem diphasischen und dem monophasischen Zustand zu registrieren. Dabei ergab sich: Das negative Potential, das nach Verschwinden der Ein- und Aus-Effekte übrigbleibt, hat dieselbe Größe in Beziehung zur Intensität der Helligkeitsreize wie das negative Zwischenpotential. Es besteht also kein Grund dagegen, anzunehmen, daß beide miteinander identisch sind.

Der Übergang vom diphasischen in den monophasischen Zustand des ERG wurde mehrfach genau verfolgt. Er ging, nachdem die Ein- und Aus-Effekte vorher eine bis mehrere Stunden lang in Beziehung zur Intensität der Lichtreize genau die gleiche Höhe behalten hatten, in einem Zeitraum von 5 bis 10 Min. vor sich. Dabei geschah die Abnahme des Ein- und des Aus-Effektes genau parallel. Diese Parallelität läßt einen inneren Zusammenhang zwischen beiden Potentialen vermuten; ein zufälliges Parallelgehen wäre bei den geschilderten Zeitverhältnissen unwahrscheinlich. Auffällig am negativen Gleichpotential ist seine eckige Form, die für ein besonders schnelles Reaktionsvermögen spricht. Hierüber wird in Abschnitt 4 Genaueres mitgeteilt. Die von AUTRUM und GALLWITZ beschriebenen negativen Potentiale von *Calliphora* nach Extirpation der optischen Ganglien reagierten weit träger.

Das Verschwinden der Ein- und Aus-Effekte hatte also keinen beobachtbaren Einfluß auf den Bestand und die Größe des negativen Zwischenpotentials. Das Gesamt-ERG (Abb. 1a, 2a, 3a) läßt sich daher formal als Summe bzw. Ueberlagerung zweier Komponenten verstehen, von denen die eine aus dem negativen Gleichpotential, die andere aus den (miteinander im Zusammenhang stehenden?) Ein- und Aus-Effekten besteht. Die formale Zerlegung eines Gesamt-ERG der Fliege *Sarcophaga* nach diesem Prinzip ist auf Abbildung 2(c) durchgeführt.

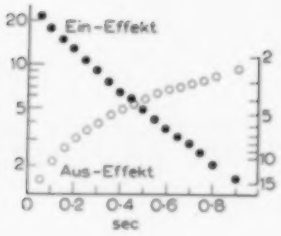
3. DAS ERG AUF UNREGELMÄßIGE HELLIGKEITSVERLÄUFE

Reizgebung: Photographien von Pflanzen-(Brennessel)-Gestrüpp wurden vergrößert auf durchsichtigen Planfilm kopiert. Die „Diapositive" wurden an einer Scheibe von 1 m Durchmesser montiert. Die Achse der Scheibe war an der Achse eines Untersetzungsgetriebes befestigt, welches von einem regelbaren Elektromotor angetrieben wurde. Das unregelmäßige Helligkeitsprogramm für das Fliegenauge wurde dadurch erzeugt, daß der Strahlengang der vorhin beschriebenen Lichtquelle in der Ebene der rotierenden Scheibe punktförmig konzentriert wurde, so daß er durch die Fortbewegung der „Diapositive" in ganz unregelmäßiger Folge erhellt und abgedunkelt wurde. Das Helligkeitsprogramm für das Fliegenauge, das auf diese Weise entstand, ist auf Abb. 4(b) abgebildet. Es wurde mit einem Photoelement an der gleichen Stelle aufgenommen, an der sich später das Fliegenauge befand. Dabei wurde die gleiche Verstärkeranordnung verwendet wie oben beschrieben.

Reaktion des negativen Gleichpotentials des ERG

Nachdem das ERG eines Auges von *Sarcophaga* monophasisch geworden war (sodaß es nur noch aus dem negativen Gleichpotential Abb. 2(b), 3(b) bestand),

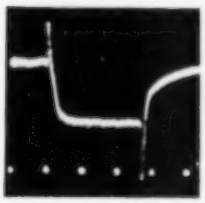
(a)



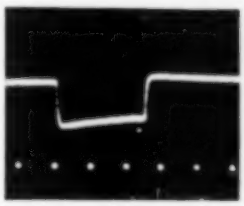
(b)

ABB. 1.

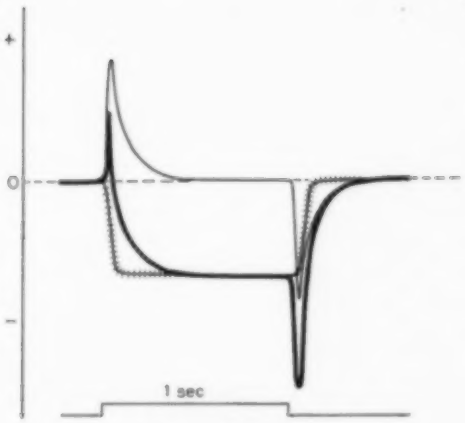
(a) ERG auf Belichtung von genau 2,5 sec Dauer; *Eristalis*-Auge.
(b) Uebertragung je eines Ein- und Aus-Effektes in halblogarithmischem Masstab. Ordinaten: Gemessenes Potential. Der linke Ordinatenmasstab gilt für den Ein-, der rechte für den Aus-Effekt. Ausgemessen wurde nicht die in Abb. (a) reproduzierte Aufnahme, sondern zwecks größerer Genauigkeit unmittelbar vorher bei höherer Verstärkung einzeln aufgenommene Ein- und Aus-Effekte.



(a)



(b)



(c)

ABB. 2.

(a) ERG auf Belichtung von etwa 1 sec Dauer; *Sarcophaga*—Auge. Zeitmarkierung: 500 msec von Punkt zu Punkt.
(b) Belichtungspotential bei gleicher Verstärkung ohne Aenderung der Versuchsanordnung zu einem späteren Zeitpunkt (2 Std. nach der vorigen Aufnahme). Zeitmarkierung wie a.
(c) Vollständiges ERG (dick ausgezogene Linie, nach Abb. a) und negatives Belichtungspotential (quergestrichene Linie, nach Abb. b) übereinandergezeichnet. Die dritte (dünne durchgezogene) Kurve, die den Charakter eines trägheitsbehafteten, zeitlichen Differentialquotienten des Helligkeitsverlaufes hat, ist errechnet durch Subtraktion der zweiten Kurve von der ersten. Die dick ausgezogene Kurve ist also die Summe der beiden dünn gezeichneten Komponenten. Am Fuss der Zeichnung: Helligkeitsverlauf des Reizlichtes.

(a) (b)

ABB. 3. Ein weiteres Beispiel entsprechend Abb. 2(a) und (b).

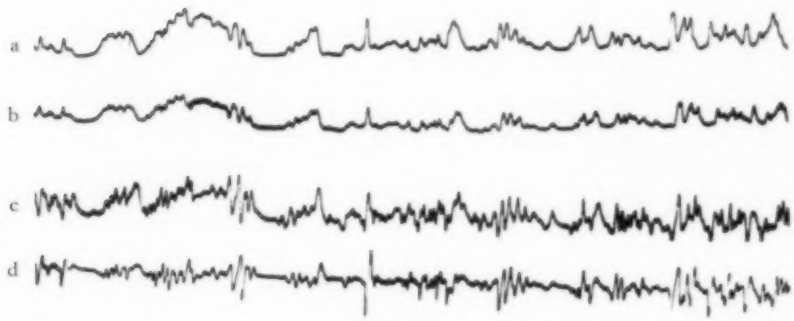


ABB. 4.

(a) Unregelmäßiger Helligkeitsverlauf, wiedergegeben durch das Belichtungspotential des Fliegenauges, von dem Abb. 2 stammt, in dem von Abb. 2(b) repräsentierten Zustand (negatives Potential).
(b) Der gleiche unregelmäßige Helligkeitsverlauf, aufgenommen mit einem Photoelement.
(c) Reaktion des gleichen Fliegenauges wie oben, aber in dem von Abb. 2(a) repräsentierten Zustand (mit funktionstüchtigen Ein- und Aus-Effekten).
(d) Reaktion des gleichen Photoelements wie oben, aber unter Zwischenschaltung eines elektrischen Differenzierngliedes.

ABB. 5. Reaktion des Systems Photozelle-Differenzierglied, mit Hilfe dessen Abb. 4(d) aufgenommen wurde, auf etwa 1 sec dauernde Belichtung. (Künstlicher „ Ein und Aus-Effekt "). Zeitmarken: 500 msec.

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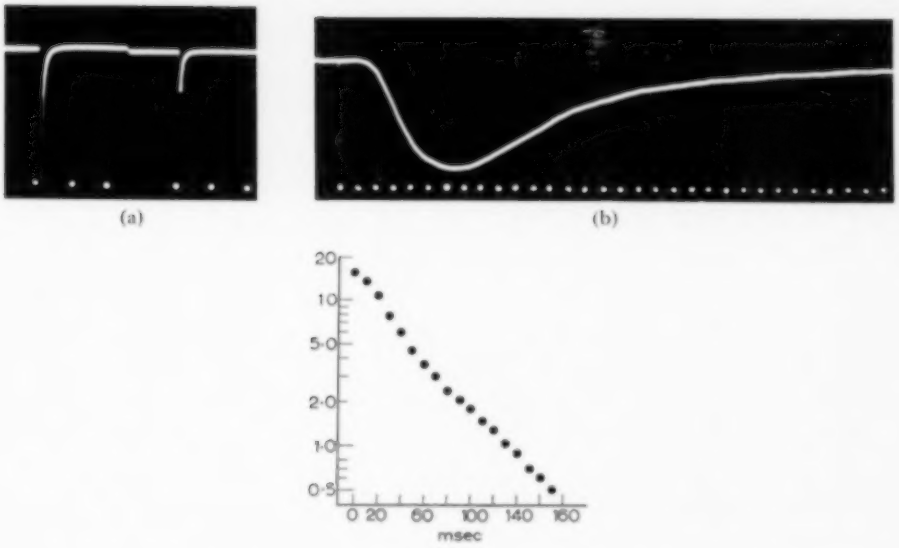

(a) (b) (c)

Abb. 6. Reaktion auf den Elektronenblitz. Auge von *Sarcophaga* in dem von Abb. 2(b) repräsentierten Zustand.

(a) bei geringer Registriergeschwindigkeit aufgenommen, rechts bei dreifach geringerer Verstärkung. Zeitmarken: 500 msec

(b) bei 50 mal so großer Registriergeschwindigkeit aufgenommen wie Abb. 6(a). Der helle Fleck markiert den Augenblick des Blitzes. Zeitmarken: 5 msec.

(c) Uebertragung des abklingenden Teils der Kurve Abb. 6(b) vom Scheitelpunkt an in halblogarithmischem Masstab.

(a) (b) (c)

Abb. 7. Reaktion des Fliegenauges in dem von Abb. 2(a) repräsentierten Zustand auf den Elektronenblitz.

(a), (b) bei geringer, (c) bei 50 facher Registriergeschwindigkeit. (a und b) von verschiedenen Tieren, (c) vom gleichen Tier wie (b). Zeitmarken: (a) und (b) 500 msec, (c) 5 msec. Der verwaschene helle Fleck markiert den Moment des Blitzes.

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wurde das Belichtungspotential auf die Reizung mit dem unregelmäßigen Helligkeitsverlauf Abb. 4(b) aufgenommen. Wie Abb 4(a) zeigt, folgte das Potential den Schwankungen der Helligkeit in prinzipiell der gleichen Weise wie die Spannung der Photozelle.

Reaktion des vollständigen ERG

Abb. 4(c) zeigt die Reaktion des gleichen Fliegenauges wie Abb. 4(a), jedoch v o r der Umwandlung des ERG vom diphasischen in den monophasischen Zustand. Erwartungsgemäss ist das Bild andersartig; die Schwankungen der Kurve sind steiler und häufiger als auf Abb. 4(a).

Um nachzuprüfen, ob diese Kurve den Differentialquotienten der Kurve Abb. darstellte, wurde die unregelmäßige Helligkeitsverteilung wie auf (Abb. 4a, b) mit dem Photoelement aufgenommen, zwischen dieses und den Verstärkereingang jedoch ein elektrisches Differenzierglied (bestehend aus einem Kondensator von 0,1 μF und einem Regelwiderstand 100 kΩ) eingeschaltet. Dadurch wurde der von der Photozelle aufgenommene Helligkeitsverlauf dem Verstärker in differenzierter Form zugeführt.

Abb. 4 (d) zeigt die Aufnahme des elektrisch differenzierten Helligkeitsverlaufs. Der Vergleich mit der Reaktion des vollständigen Fliegen-ERG (Abb. 4c) offenbart die prinzipielle Gleichheit beider Kurven. Ein Unterschied besteht nur darin, daß im Fliegen-ERG die Grundlinie gemäß der jeweiligen Grundhelligkeit schwankt, was bei der Modellkurve (Abb. 4d) nicht der Fall ist. Dieser Unterschied erklärt sich sofort beim Vergleich von Abb. 2(a) und 5. In Abb. 5 ist die Reaktion des Systems Photoelement-Differenzierglied auf etwa 1 sec dauernde Belichtung wiedergegeben. Es ergibt sich das Bild eines künstlichen Ein- und Aus-Effektes. Der Modellkurve fehlt jedoch das negative Gleichpotential, das beim Fliegenauge mit abgeleitet wird. Der Unterschied zwischen Abb. 2(a) und 5 erklärt damit anschaulich die Unterschiedlichkeit von Abb. 4(c) und 4(d).

Die Versuche dieses Abschnitts zeigen, daß Ein- und Aus-Effekt darin zusammenwirken, ein physiologisches Resultat hervorzubringen, das dem Differentialquotienten des primär wahrgenommenen Helligkeitsverlaufes entspricht. Diese Tatsache könnte insofern Interesse gewinnen, als das Sehen von Bewegungen bei Insekten (nicht unbedingt beim Menschen!) auf der Auswertung von Helligkeits*änderungen* beruht; dies setzt eine physiologische Differentiation voraus.

4. DAS ERG AUF REIZUNG DES AUGES MIT DEM ELEKTRONENBLITZ

Der Elektronenblitz hat als Lichtreizquelle zwei Eigenschaften, die es erhoffen ließen, zusätzliche Information über das beschriebene funktionelle Geschehen zu gewinnen: Die kurze Zeitdauer seiner Einwirkung, die in Bezug auf die Zeitkonstanten des Auges einem „Nadelimpuls" gleichkommt, und die hohe Lichtintensität. Verwendet wurde der Elektronenblitz BRAUN-HOBBY. Der Reflektor war in 80 cm Entfernung vom Auge angebracht. Die Reizung erfolgte mit der am Gerät einstellbaren „halben Intensität." Der Moment des Blitzes bildete sich auf dem Registrierstreifen direkt optisch ab.

Reaktion des negativen Gleichpotentials des ERG

Die Reaktion auf einen Elektronenblitz zeigt Abbildung 6 bei Registriergeschwindigkeiten von (a) 1,02 cm/ sec und (b) 51 cm/ sec, für ein Fliegenauge, dessen ERG die Ein- und Aus-Effekte bereits verloren hat und nur noch aus der negativen Komponente besteht. Die Bilder zeigen Folgendes: 6 bis 8 msec nach dem Reiz beginnt ein sehr steiler negativer Anstieg des Potentials, der nach 30 msec beendet ist. Das Abklingen des Potentials dauert ungefähr 120 msec. Die Reaktion dauert also viel länger als der Blitz, dessen Leuchtzeit nach Angabe der Hersteller 1 msec beträgt. In derselben Weise wie oben wurde auch bei dieser Reaktion die Form der Abklingkurve untersucht. Abb. 6(c) zeigt, daß auch diese Kurve nach Übertragung auf halblogarithmisches Papier nahezu eine Gerade bildet. Auch hier handelt es sich also um die mathematisch einfachste Abklingfunktion, die die Mitwirkung nur einer Art von Energiespeichern vermuten läßt. Wegen ihres exponentiellen Abklingens läßt sich auch auf diese Kurve der Begriff der Zeitkonstanten anwenden: Die Zeitkonstante des Abklingens der negativen Komponente des ERG der Fliege Abbildung 6(b) ist 55 msec. Wie man daran sieht, ist die Trägheit des negativen Potentials geringer als die der Ein- und Aus-Effekte. Dies war schon wegen der eckigen Form der Reaktionskurve Abb. 2(b) vermutet worden.

Reaktion des vollständigen ERG

Wenn fast gleichzeitig drei Komponenten—negatives Gleichpotential, Ein-Effekt und Aus-Effekt—durch einen nur 1 msec dauernden Lichtreiz ausgelöst werden, so ist zu erwarten, daß ein ganzer Komplex sich summierender Einzelfunktionen auftreten wird. Abb. 7 zeigt die Reaktionen des vollständigen ERG, wiederum mit zwei verschiedenen Registriergeschwindigkeiten aufgenommen. Wie man sieht, bestätigte sich im Vergleich zu Abb. 6 die komplexe Natur der Reaktion: Zunächst erfolgt rund 4 msec nach dem Elektronenblitz eine positive Auslenkung. Sie kann nur Ausdruck des positiven Ein-Effektes sein. Überraschend ist, daß dieser Teil des ERG nach kürzerer Latenzzeit in Erscheinung tritt, als die negative Gleichspannungskomponente. Diese erscheint wie im isolierten Zustand rund 7 m sec nach dem Reiz; sie macht sich hier dadurch bemerkbar, daß der positive Anstieg des Ein-Effektes abgebrochen wird und das ERG sehr schnell in eine negative Phase übergeht. Ein dritter Abschnitt des ERG setzt etwa in dem Augenblick ein, wo die isolierte negative Komponente—siehe Abb. 6—zur Null-Linie zurückzukehren beginnen würde. Dies geschieht jedoch bei der Abb. 7 nicht, sondern es schließt sich ein zweiter Abschnitt eines negativen Potentiales an, der jedoch langsamer zunimmt als der erste. Sein Verlauf ist merkwürdig unregelmäßig. Nach dem Zeitverhältnis und der offenbar größeren Trägheit wird es sich hierbei um eine Auswirkung des Aus-Effektes handeln.—Erst danach kehrt das Potential langsam zur Null-Linie zurück. Im ganzen ist die Reaktion viel träger als die des negativen Gleichpotentials (Abb. 6) allein.—Vor Erreichung der Null-Linie zeigt sich eine weitere Unstetigkeit der Reaktionskurve: Das Zurückgehen erfolgt nicht gleichmäßig, sondern wird vorübergehend

vollständig oder beinahe vollständig unterbrochen. Ob diese Unstetigkeit auf einem der drei schon diskutierten Elemente des ERG beruht oder einer vierten Komponente entspringt, vermag ich nicht anzugeben.

Aus dem Gesamt-ERG nach Reizung mit dem Elektronenblitz läßt sich folgendes ableiten: Da der Ein-Effekt deutlich früher erscheint als das negative Gleichpotential, ist es kaum denkbar, daß er die kausale Folge des letzteren ist. Das umgekehrte Verhältnis—das Gleichpotential als kausale Folge des Ein-Effektes—ist aus einem andern Grunde unwahrscheinlich: weil sich das negative Gleichpotential völlig unverändert auslösen läßt, auch nachdem das System der Ein-Aus-Effekte zu funktionieren aufgehört hat (Abb. 2b, 3b). Nach den gewonnenen Versuchsergebnissen scheinen das negative Gleichpotential und das System Ein-Aus-Effekt voneinander unabhängige Folgen des Lichtreizes zu sein, die sich nur durch die Art der Ableitung zum Gesamt-ERG überlagern.

5. DISKUSSION UND ZUSAMMENFASSUNG

Unter den beschriebenen Versuchsbedingungen verloren sich regelmäßig 1 bis 3½ Stunden nach Abtrennen des Fliegenkopfes Ein- und Aus-Effekt des Elektroretinogramms und hinterliessen ein rein negatives Belichtungspotential von der Höhe des vorherigen negativen Zwischenpotentials (Abb. 2b, 3b). Der zeitliche Verlauf dieser Umwandlung spricht für eine *Beziehung* zwischen Ein- und Aus-Effekt, für größere gegenseitige *Unabhängigkeit* zwischen dem System „Ein- und Aus-Effekt" einerseits und dem negativen Zwischenpotential andererseits.

Bei Ein-Effekt und negativem Potential ergaben sich mit guter Annäherung exponentielle Abklingvorgänge.

Trägheitskonstanten waren beim negativen Potential 55 msec, bei einem Ein-Effekt 350 msec.

Ein- und Aus-Effekt zusammen bringen ein Belichtungspotential hervor, das formal dem Differentialquotienten des Helligkeits-Reiz-Programms entspricht; das negative Gleichpotential dagegen gibt den Absolutwert der Helligkeit wieder. Man kann das ERG von *Sarcophaga* daher *formal* als Summe aus einem differenzierten und einem undifferenzierten Anteil betrachten.

Weitergehende theoretische Schlüsse als diese Angaben über formale Zusammenhänge zwischen den gemessenen Größen—also Zeitabstände, zeitliche Verläufe und relative Intensitäten—erscheinen mir beim augenblicklichen Stand unserer Kenntnisse über die diphasischen Belichtungspotentiale der Insekten verfrüht.

Einige der verwendeten Apparate sind Leihgaben der Deutschen Forschungsgemeinschaft an Herrn Prof. Dr. F. P. MÖHRES, Direktor des Zoophysiologischen Instituts der Universität Tübingen.

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ÜBER DIE GEWEBSATMUNG DES KARTOFFELKÄFERS *LEPTINOTARSA DECEMLINEATA* (SAY) IN DER WINTERRUHE UND IM WACHZUSTAND

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Abstract The possibility of the existence of a diapause hormone in the beetle *Leptinotarsa decemlineata* has been investigated. The experimental results provide support for the following assumptions all of which, however, require confirmation by other methods: The corpora allata are activated during the end of diapause and to some extent as the season proceeds. The corpora allata hormone is stored at low temperatures but it is immediately liberated as diapause ends. The effect of this hormone is especially great in the posterior part of the body. The inhibition of metabolism during diapause is brought about by an inhibitory hormone that probably circulates in the blood, and its effects are therefore demonstrable in both anterior and posterior parts of diapausing animals. The inhibitory hormone is probably not stored. It may perhaps be produced in both the anterior and posterior parts of the body, for instance by both the suboesophageal ganglion and other ganglia of the ventral chain. During the end of diapause, production of the inhibitory hormone either ceases or its effect is suppressed by the hormone from the corpora allata.

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Über die hormonale Seite der Diapause von Insektenentwicklungsstadien ist besonders im letzten Jahrzehnt viel gearbeitet worden (vergl. die zusammenfassenden Darstellungen von Agrell, 1951; Williams, 1951; Andrewartha, 1952; Pflugfelder, 1952; Wiggflsworth, 1953; Lees, 1955). Die Hemmung der bekanntlich hormonal gesteuerten Entwicklung stand bei diesen Untersuchungen im Vordergrund. Die besonderen Probleme der Winterruhe erfaßt man besser an erwachsenen Insekten, da hier die Entwicklungshemmung mit ihrer besonderen Problematik entfällt bzw. sich nur auf die Gonaden erstreckt. Beim Kartoffelkäfer findet die Ovulation während des ganzen adulten Lebens statt, doch ist die Ausbildung fertiger Eier in der Winterruhe unterbrochen (De Wilde, 1954). Manche Autoren haben diese spezielle Entwicklungshemmung untersucht, doch ist fraglich, ob man damit das Wesentliche des imaginalen Ruhestadiums erfaßt hat. Berechtigt wäre eine solche Beschränkung, wenn die eine Gonadenentwicklung beeinflussenden Faktoren (z. B. Hormone) auch gleichzeitig die Aenderung des Verhaltens und die von uns untersuchten, tiefgreifenden Stoffwechselumstellungen mitbedingen würden (vergl. Marzusch, 1952; Precht, 1953; Carlsen, 1953).

* Teil einer Dissertation (Anregung und Anleitung: Prof. Dr. H. Precht). Für die Beschaffung von Kartoffelkäfern danke ich den Herren Dr. W. Holz, Pflanzenschutzamt Oldenburg i.O. und Dr. Hülsenberg, Pflanzenschutzamt Frankfurt a.M. Einen Teil der benutzten Apparate stellte die Deutsche Forschungsgemeinschaft zur Verfügung.

Es interessieren hier vor allem die (allerdings noch wenigen) Autoren, die für diapausierende Entwicklungsstadien ein spezielles Diapausehormon annehmen. Besonders HINTON (1954) hat sich für die Existenz eines solchen Hormons auf Grund der Befunde an Lepidopteren von WILLIAMS (1951), FUKUDA (1953) und HASEGAWA (1951) u. a. eingesetzt (vergl. jedoch die Kritik von LEES, 1955, S. 118). Das Hormon soll unter der Kontrolle des Gehirns vom Unterschlundganglion sezerniert werden. Die Corpora allata werden durch dieses Inkret gehemmt, der Stoffwechsel wird durch eine Ausschaltung des Cytochromsystems der meisten Organe herabgesetzt. — An dem Sauerstoffverbrauch von Homogenisaten der winterruhenden Kartoffelkäfer war das sauerstoffübertragende Ferment beteiligt (PRECHT, 1953; vergl. auch LUDWIG, 1953 a, b). Das Hormon der Prothorakaldrüsen wirkt nach HINTON dem Diapausehormon entgegen. Jenes Hormon dürfte jedoch für die Beendigung der Winterruhe unseres Versuchstieres keine Rolle spielen, weil die Prothorakaldrüsen sich bei den meisten langlebigen erwachsenen Insekten zurückbilden. Das Diapausehormon wird nach HINTON sezerniert, nachdem das Gehirn eine Beeinflussung durch Faktoren erfahren hat, die eine Winterruhe auslösen. Als solche kommen mehrere Außenfaktoren in Frage, beim Kartoffelkäfer besonders die Tageslänge (DE WILDE, 1954; GRISON, 1954). DE WILDE (1953) konnte bei der Hälfte der Käfer durch geeignete Bedingungen die Winterruhe unterdrücken, doch weisen andere Autoren darauf hin, daß auch gewisse Anzeichen für innere auslösende Faktoren im Sinne einer obligatorischen Ruhe vorliegen (vergl. FABER, 1949). Das Gehirn würde nach diesen Vorstellungen die Corpora allata auf dem Umwege über das Diapausehormon hemmen. Dies könnte auch die Drosselung des Stoffwechsels bedingen bzw. mitbedingen.

Es erhebt sich aber gleich die Frage, ob die Annahme eines solchen Diapausehormons überhaupt noch notwendig ist, da die Herabsetzung des Stoffwechsels auch durch die Reduzierung des Hormons der Corpora allata erklärt werden kann, wofür die Versuche von THOMSEN (1949) an *Calliphora* sprechen.

Der Anstoß zum Wiedererwachen kann vom Gehirn ausgehen. Implantierte Gehirne sind dazu in der Lage (GRISON, 1949). Es ist zu berücksichtigen, daß beim Kartoffelkäfer nicht tiefe sondern hohe Temperaturen die Ruhe brechen. Nach DE WILDE (1954) sollen die dann aktivierten und heranwachsenden Corpora allata ein gonadotropes Hormon ausscheiden. Es ist noch fraglich, ob dieses Hormon die Stoffwechselsteigerung mitbedingt, da auch die innersekretorischen Drüsen der Wirbellosen u. U. mehrere Hormone produzieren. Eventuell spielen die Corpora cardiaca dabei noch eine Rolle.

PRECHT (1953, S. 338) stellte fest, daß eine Mischung von Homogenisaten von schlafenden und erwachten Kartoffelkäfern einen geringeren Sauerstoffverbrauch aufweist als der errechnete Mittelwert von beiden Einzelsuspensionen. Diese Versuche sind mit Teiltieren weiter ausgedehnt worden, um Rückschlüsse auf die hormonale Seite der Winterruhe machen zu können, wobei besonders die Frage interessiert, ob die Existenz eines Diapausehormons wahrscheinlich ist.

MATERIAL UND METHODEN

Die Kartoffelkäfer wurden im August und September in den Gebieten um Frankfurt a.M., Oldenburg i.O. und Neumünster (Schl.-Holst.) gesammelt. Die Käfige zur Aufbewahrung der Tiere standen in einem ungeheizten, feuchten Kellerraum. Der Erdboden (Zusammensetzung: ¼ Sand, ¼ Kies und ½ Torfmull) wurde alle 14 Tage auch während des Winterschlafs der Tiere angefeuchtet. Bis Anfang Oktober hatte sich der größte Teil eingegraben; nur diese Käfer kamen für die Versuche in Frage. Vor dem Winterschlaf diente Kartoffelkraut als Futter, aufgewachte Tiere sind nie gefüttert worden. Die Anpassung an konstante Temperaturen fand in Blumentöpfen mit derselben Erde wie in den Käfigen bei 100%iger Luftfeuchtigkeit und Dunkelheit statt. Als Unterscheidungsmerkmale der Geschlechter dienten die Unterschiede im letzten sichtbaren Sternum des Abdomens. Die Dauer der Anpassung betrug stets 9–10 Tage. Einige Tiere verließen, durch die Umbettung gestört, während der Temperaturanpassung die Erde. Sie fanden nur zu den Versuchen Verwendung, wo dies ausdrücklich vermerkt ist.

Die Atmungsmessungen erfolgten mit Hilfe der WARBURG-Apparatur. Die Käfer wurden nach Entfernung der Elytren entweder mit einem Mikrotommesser fein zerhackt oder in einer Glasmühle (nach POTTER und ELVEHJEM) gemahlen. — Im ersten Fall gelangten in jeden Trog: 270–280 mg Hackbrei, 1 cm³ Phosphatpuffer (p_H6,8; ^{m}/15) und 1 cm³ ^{m}/10-Natriumsuccinatlösung. Die Versuchstemperatur betrug 25° C. Alle Werte sind in O_2-Verbrauch pro Gramm und Stunde angegeben. — Im zweiten Fall enthielten die hergestellten Gewebesuspensionen pro Volumeneinheit stets die gleiche Gewebemenge; z. B. kamen auf 2 g Gewebe immer 7 cm³ Phosphatpuffer (p_H6,8; ^{m}/15) und 7 cm³ ^{m}/10-Natriumsuccinatlösung. Die Versuchstemperatur betrug 30° C. Die Werte zeigen den O_2-Verbrauch in mm³ pro 2 cm³ Suspension und Stunde an.

Zur statistischen Sicherung der Atmungsunterschiede bei den Gewebesuspensionen nach dem üblichen t-Test wurde die von CSIK und WOLSKY (1939) verwendete t-Formel für diesen besonderen Fall umgewandelt (P_1-Werte):

$$\frac{\frac{\bar{y}+\bar{z}}{2}-\bar{x}}{\sqrt{\left(\frac{s^2_y+s^2_z}{4(n_y \cdot n_z-1)}+\frac{s^2_x}{n_x-1}\right)}}=t \qquad n_y \cdot n_z+n_x-2=m \text{ (Freiheitsgrade)}$$

$\bar{y}, \bar{z}, \bar{x}$ = Mittelwerte aus den Einzelmessungen; y und z = Stammesuspensionen, x = Mischsuspension.

$s^2 = \frac{\Sigma \Delta u^2}{n}$ Δu = Abweichungen der Einzelmessungen vom Mittelwert
n = Anzahl der Messungen

Im übrigen fand die t-Formel von PAETAU (1943) Anwendung (P_2-Werte).

1. DER EINFLUSS DER WINTERRUHE AUF DEN SAUERSTOFFVERBRAUCH VON GEWEBEN AUS GANZEN KÄFERN

Zu diesen Versuchen wurde entsprechend der Methode von MARZUSCH (1952) Hackbrei von Ganztieren verwandt. Nach MARZUSCH zeigt der Sauerstoffverbrauch des Gewebes wie der von intakten, winterruhenden Kartoffelkäfern eine Temperaturadaptation nach Typ 3, d. h. die Meßwerte fallen bei gleichen Versuchsbedingungen, wenn die Tiere vor dem Versuch an eine höhere Temperatur adaptiert wurden. Für intakte, schlafende Käfer konnte dies Ergebnis bestätigt werden (Ausmaß der Adaptation etwa 30%). Der Adaptationseffekt im Sauerstoffverbrauch des Gewebes war aber im Gegensatz zu den Ergebnissen von M.

bedeutend geringer (Tab. 1 und Abb. 1, I–III). Interessant ist, daß sich zu Beginn der Winterruhe auch die an 24° adaptierten Tiere einordnen.* Zu einer späteren Jahreszeit führt diese hohe Temperatur zu einer Steigerung des

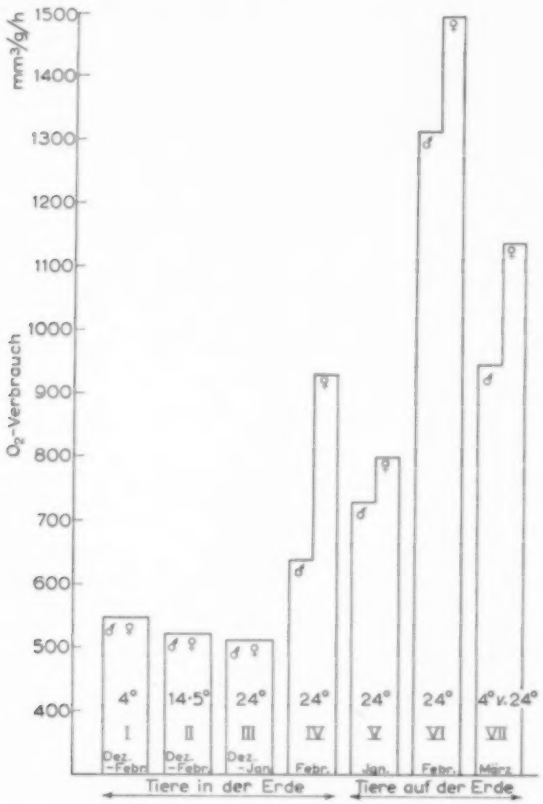


ABB. 1. Der Sauerstoffverbrauch des Hackbreies aus unterschiedlich adaptierten, schlafenden und wachen Kartoffelkäfern. In den Säulen sind angegeben: unten die Zeit der Messung, in der Mitte die Adaptationstemperatur und Nr., oben die Geschlechter.

Stoffwechsels. Dieses Erwachen kann sich auch schon bei Tieren zeigen, die noch im Boden liegen (IV), wiederum ein Beweis dafür, wie wenig die Bewegungen der Tiere Aussagen über ihren inneren Zustand gestatten (vgl. PRECHT, 1953).

Die Wärmebehandlung wirkt sich auf die Weibchen mehr aus als auf die Männchen und bei beiden mit fortschreitender Jahreszeit immer stärker. Eine Umsetzung der im Februar untersuchten, bei 24° aufbewahrten, aufgewachten Tiere auf 4° für die Dauer von 10 Tagen macht den Aufwacheffekt nicht wieder

* Bei den ersten Werten (I–III) sind Männchen und Weibchen zusammengefasst worden, da sich keine Unterschiede zeigten.

rückgängig. Der Abfall des Sauerstoffverbrauchs hängt zumindest z. T. mit dem fortdauernden Hungerzustand zusammen (VII).

TABELLE 1. Der Sauerstoffverbrauch des Hackbreies aus unterschiedlich adaptierten, schlafenden und wachen Kartoffelkäfern. Adaptation in Blumentöpfen bei 100% Luftfeuchtigkeit. Statistische Sicherungen: I/II, $P_2 = 0{,}035$; I/III, $P_2 = 0{,}0015$. In klammern Zahl der Messungen.

Aktivitätszustand der Tiere	Nr.	Adapt.-Temp. in °C	Zeit der Messung	mm³ O_2-Verbrauch pro g und Std.
schlafend	I	4	Dez.-Febr.	♂+♀ 546 ± 7,4 (12)
	II	14,5	Dez.-Febr.	♂+♀ 521,5 ± 8,0 (12)
	III	24	Dez.-Jan.	♂+♀ 511 ± 6,0 (11)
	IV	24	Febr.	♂ 638 ±25 (3)
				♀ 930 ±35 (3)
wach	V	24	Jan.	♂ 729 ±36 (3)
				♀ 799 ±24,5 (3)
	VI	24	Febr.	♂ 1 312 ±67,5 (3)
				♀ 1 495 ±53 (3)
	VII	4 (vorher 24)	März	♂ 945 ±35,5 (3)
				♀ 1 036 ±27 (3)

Die Kartoffelkäfer sind kein sehr günstiges Untersuchungsobjekt für die Temperaturadaptation. Bei Aufbewahrung in mittleren Temperaturen überlagert sehr leicht ein gewisser Aufwacheffekt die Drosselung des Stoffwechsels durch die Adaptation (vgl. PRECHT, 1953, Tab. 1). KIRBERGER (1953) beobachtete das gleiche bei Weinbergschnecken, wobei sich die einzelnen Organe sogar noch unterschiedlich verhalten konnten. — Es kommt eine weitere Fehlerquelle hinzu. Man muß stets darauf achten, daß bei Verwendung von Hungertieren die als Adaptationseffekt gedeutete Drosselung des Stoffwechsels nicht durch den Hungerzustand selbst bedingt wird, der natürlich bei höheren Anpassungstemperaturen stärker in Erscheinung tritt als bei niederen. Dies gilt für die Atmung der Gewebe besonders dann, wenn ganze Tiere gewogen werden, weil der wohl unabhängig von der Adaptation konstant bleibende Anteil des Chitins bei den stärker hungernden warmadaptierten Käfern auf Kosten des atmenden Gewebeanteils höher ist als bei kaltadaptierten Käfern. Der Adaptationseffekt wurde deshalb nicht weiter untersucht.

2. MISCHUNG VON GEWEBESUSPENSIONEN

Die verwendeten Suspensionen enthielten gemahlenes Gewebe von Köpfen und Prothorax (*CP*), Thorax und Abdomen (*TA*) und Meso- u. Metathorax und Abdomen (*MA*). Bei den Abkürzungen wird die Adaptationstemperatur davorgesetzt; 5*MA* bedeutet demnach eine Suspension aus Meso-, Metathorax und

Abdomen von Käfern, die bei 5° aufbewahrt wurden und bei dieser Temperatur bewegungslos in der Erde lagen. Es wurde der Sauerstoffverbrauch dieser einzelnen Suspensionen und der von Mischsuspensionen gemessen.

Abb. 2 und Tab. 2 geben den Sauerstoffverbrauch der Suspensionen *CP* und *MA* wieder. Bei schlafenden Tieren verbraucht das Hinterende weniger Sauerstoff als der Vorderteil. Die mit fortschreitender Jahreszeit zunehmende Stoffwechselsteigerung durch eine Wärmebehandlung betrifft aber im wesentlichen das Hinterende; vermutlich „erwacht" in stoffwechselphysiologischer Hinsicht besonders das Abdomen. Der Aufwacheffekt ist bei den Weibchen bis zum Februar stärker als bei den Männchen. Im März ist dieser Unterschied nicht mehr vorhanden. Der Hungerzustand verstärkt sich natürlich mit fortschreitender Jahreszeit. Das dürfte generell zu einer Abnahme der Atmungswerte führen, wenn diese auch durch die Stoffwechselsteigerung beim Aufwachen überdeckt wird. Der Hungereffekt wird sich zu der späten Jahreszeit bei den Weibchen mehr auswirken als bei den Männchen, da ihre gesteigerte Atmung die Reserven mehr erschöpft.

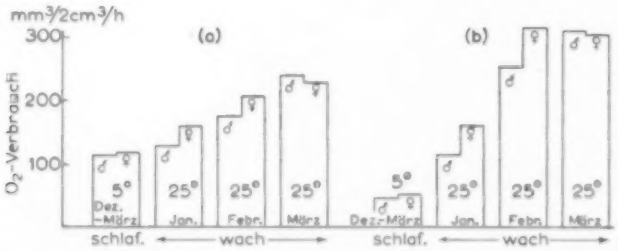


ABB. 2. Der Sauerstoffverbrauch von (a) Kopf-Prothorax-Suspensionen (*CP*) und (b) Abdomen-Meso-, Metathorax-Suspensionen (*MA*) von schlafenden und wachen Kartoffelkäfern. In den Säulen sind angegeben: unten die Zeit der Messung, in der Mitte die Adaptationstemperatur, oben die Geschlechter.

Die verhältnismäßig geringe Zunahme der Atmung der *CP*-Suspensionen spricht dafür, daß die Homogenisierung der immer mehr heranwachsenden und an Aktivität zunehmenden Corpora allata, wenn überhaupt, so doch nur einen geringen Effekt hat. Das Zermahlen einer Hormondrüse braucht nicht unbedingt einen Effekt zu haben, da eine Hormonvermehrung nur bewirkt wird, wenn die Drüse wirksames Hormon speichert. Auch aus den Versuchen von THOMSEN (1949) geht nicht hervor, daß eine solche Speicherung in den C. allata stattfindet. Nimmt man dennoch eine Hormonvermehrung durch das Zermahlen an, dann wirkt sich diese jedenfalls nicht in stärkerem Maße auf den Sauerstoffverbrauch der Vorderteile aus.

Wird sich ein durch Zermahlen u. U. freigesetztes Hormon überhaupt im Versuch auswirken können? Eine Hormonwirkung auf isolierte Gewebe ist oft schwer nachweisbar. Dies gilt auch für das Schilddrüsenhormon, dessen stoffwechselsteigernde Wirkung bei höheren Wirbeltieren außer Frage steht.

Lediglich HAARMANN (1936) konnte unter Anwendung der richtigen Versuchsbedingungen und Hormonkonzentrationen eine Wirkung des Thyroxins auf die

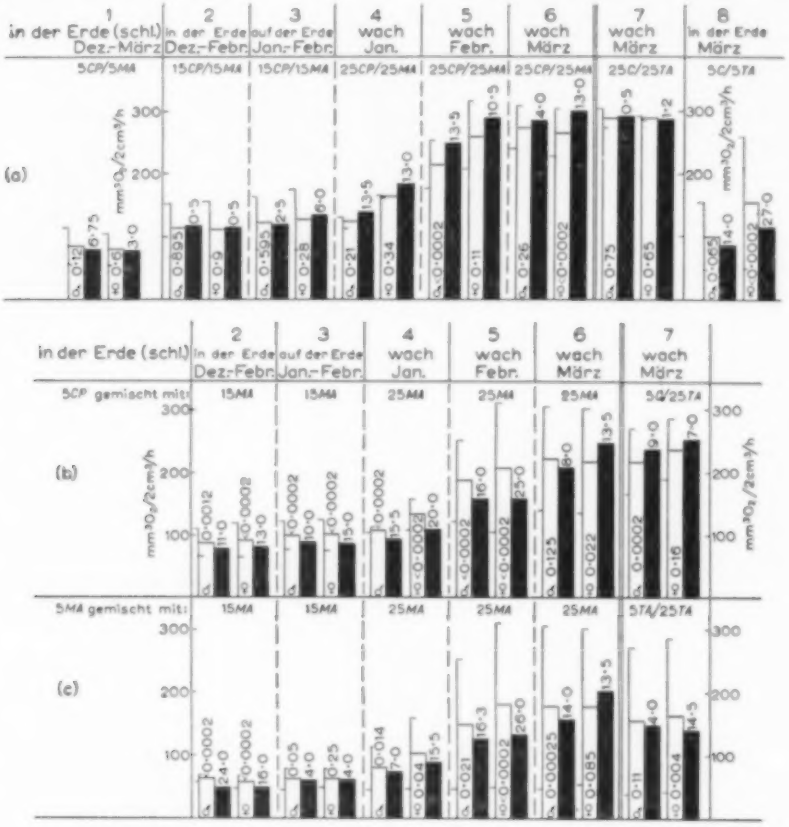
TABELLE 2. Der Sauerstoffverbrauch von Kopf-Prothorax-Suspensionen (*CP*) und Meso-, Metathorax u. Abdomen-Suspensionen (*MA*) von Kartoffelkäfern in Abhängigkeit von dem Aktivitätszustand, von der Adaptattionstemperatur und der Zeit der Messung. Adaptation in Blumentöpfen bei 100% Luftfeuchtigkeit. In klammern Zahl der Messungen.

Aktiv.-zustd. d. Tiere	Adapt.-Temp. in °C	Zeit der Messung	Sex.	CP-Susp. mm^3 O_2-Verbr./ 2 cm^3/Std.	MA-Susp. mm^3 O_2-Verbr./ 2 cm^3/Std.
schlaf.	5	Dez.-März	♂	116 ± 2,9 (43)	48 ± 1,6 (46)
			♀	118 ± 2,9 (48)	51 ± 1,8 (47)
wach	25	Jan.	♂	129,5 ±10,7 (6)	114,5 ± 5,2 (5)
			♀	161 ±13,5 (6)	160 ±15,4 (6)
		Febr.	♂	175 ±17,3 (6)	252,5 ±16,1 (6)
			♀	206 ±16,6 (6)	312 ±17,5 (6)
		März	♂	239 ± 7,0 (6)	306,5 ±16,1 (6)
			♀	226,5 ± 6,7 (6)	302 ±11,2 (6)

Sauerstoffaufnahme der Gewebe verschiedenster Tiere nachweisen. (Über weitere Wirkungen auf Mitochondrien usw. vgl. EDLBACHER-LEUTHARDT, Lehrb. d. Physiol. Chemie 1955, S. 613.) Darum muß man entsprechend auch mit einem Effekt des beim Zermahlen freigesetzten Hormons der C. allata rechnen.

Nach mehreren Autoren ist die stoffwechselsteigernde Wirkung des Thyroxins sehr viel leichter nachzuweisen, wenn die Atmung des Gewebes von Normaltieren mit der von solchen Individuen verglichen wird, die vor dem Versuch mit dem Hormon behandelt wurden (vgl. v. BUDDENBROCK, 1950, S. 27). Es handelt sich also um eine Nachwirkung der Hormonbehandlung. Solche nachwirkenden Einflüsse sind auch beim C.-allata-Hormon anzunehmen. Sie können sich evtl. nur in einem Körperteil (so in den Abdomen der Hinterenden, Abb. 2) zeigen und darauf beruhen, daß die Nährstoffe in eine leicht angreifbare Form überführt werden. Sicherlich ist die von PRECHT (1953) festgestellte Aktivierung bzw. Vermehrung der Zellfermente als eine solche Nachwirkung anzusehen, die sich besonders im Abdomen der aufgewachten Käfer bemerkbar macht. Bei Hungertieren können die hinsichtlich ihrer Synthese auf eine Vitaminzufuhr angewiesenen Zellfermente nicht vermehrt sondern höchtens aktiviert werden (vgl. CARLSEN, 1953). Schließlich mögen als weitere Folgeerscheinung einer gesteigerten Hormonproduktion der Corpora allata die mit dem Aufwachen verstärkt in den Gonaden ablaufenden Reifungsprozesse zu einem vermehrten Sauerstoffverbrauch führen.

In Abb. 3 sind die Ergebnisse von Mischungsversuchen dargestellt. Die an 15° angepaßten Käfer lagen teils auf der Erde, teils befanden sie sich beim Versuchsbeginn noch in der Erde. Da der Stoffwechsel verhältnismäßig hoch war, handelt es sich wohl in beiden Fällen in stoffwechselphysiologischer Hinsicht



(See page 139 for caption)

nicht mehr in vollem Sinne um schlafende Tiere. Es wurde sowohl der Sauerstoffverbrauch von je 2 cm³ der Einzelsuspensionen als auch der der Mischsuspensionen aus je 1 cm³ beider Einzelhomogenisate bestimmt. In Abb. 3 stellt die weiße, linke Säule jeweils den errechneten theoretischen Mittelwert aus den 2 Stammsuspensionen,* die rechte, schwarze den wirklichen Meßwert der Mischsuspension dar. Die Unterschiede sind (in %) über den schwarzen Säulen angegeben (der theoretische Mittelwert wurde gleich 100% gesetzt). Das hier

* Die Ausgangswerte sind durch zwei Markierungsstriche auf einer Geraden neben der weissen Säule wiedergegeben, von denen der nach links weisende die Atmung der links in der Tabelle angegebenen Suspension und der nach rechts weisende Strich die Atmung der rechts angegebenen Suspension bedeutet.

notwendige Absicherungsverfahren ist auf *S.* beschrieben worden; die P_1-Werte befinden sich in den weißen Säulen.

Es ist erstaunlich, daß sich bei den erwachten Käfern ein deutlicher Unterschied ergibt, wenn die *CP*- und *MA*-Suspensionen von gleich vorbehandelten

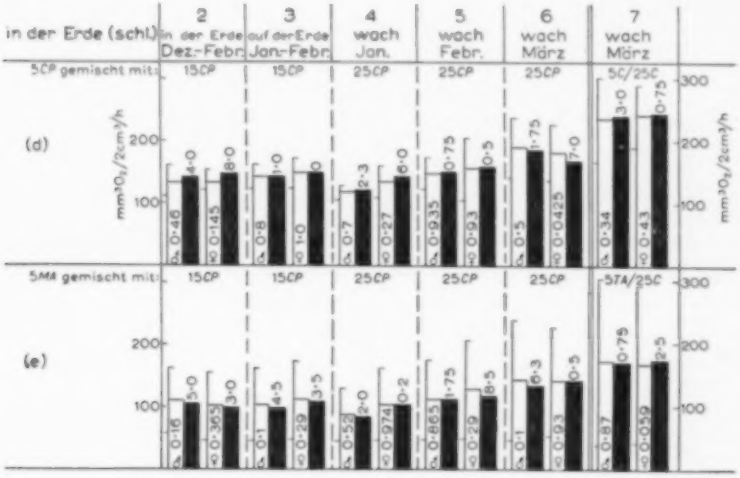


ABB. 3. Der Sauerstoffverbrauch von Mischsuspensionen (schwarze Säulen) im Vergleich zum errechneten theoretischen Atmungsmittelwert der entsprechenden zwei Stammsuspensionen (weisse Säulen) aus Kartoffelkäferteilen mit verschiedenem Aktivitätszustand. — Die Werte der zwei Stammsuspensionen sind durch zwei Markierungsstriche auf einer Geraden neben der weissen Säule wiedergegeben, von denen der nach links weisende Strich die Atmung der links in der Abb. angegebenen Suspension und der nach rechts weisende Strich die Atmung der rechts angegebenen Suspension bedeutet. Ueber den schwarzen Säulen stehen die Unterschiede in % (weisse Säule = 100%). Die weissen Säulen enthalten unten die Angabe des Geschlechts, darüber die statistische Sicherung des Unterschieds (P_1-Werte). Eingangs der Spalten 1–8 sind der Zustand der Tiere und die Zeit der Messung angeführt. — *5CP* bedeutet eine Suspension aus Kopf und Prothorax der schlafenden, an 5° C adaptierten Tiere, *25MA* bezeichnet eine Suspension aus Meso-, Metathorax und Abdomen der wachen, an 25° C adaptierten Tiere usw. Suspensionen mit der Bezeichnung *C* bestanden nur aus Köpfen und solche mit der Bezeichnung *TA* aus Thorax und Abdomen. Die Mischsuspension enthielt je 1 cm³ der zwei Stammsuspensionen.

Tieren gewonnen werden (Reihe a, Spalten 2–6). Eine Abweichung vom theoretischen Mittelwert kann dadurch zustandekommen, daß sich das durch das Mahlen im Vorderteil vermehrt in Freiheit gesetzte Hormon der C. allata vor allem in den Hinterenden auswirkt. Oder aber es handelt sich um keine Wirkung von vermehrtem Hormon während des Versuchs sondern wiederum um eine Nachwirkung des Inkrets der C. allata. Es kann, wie erwähnt, besonders in dem Abdomen eine Vermehrung der Zellfermente stattgefunden haben. Die durch Homogenisierung des Gewebes in Freiheit gesetzten überschüssigen Fermente wirken sich in der Mischsuspension dann auch auf das homogenisierte Vorderende aus.

In weiteren Versuchen sind Suspensionen von Teilen unterschiedlich vorbehandelter Käfer gemischt worden, wobei besonders die Versuche interessieren, bei denen ein Körperteil von einem einwandfrei schlafenden und der andere von einem erwachten Käfer stammt (also Käfer der Adaptationstemperaturen 5° und 25°).

Die Mischung von 5*CP*-Suspensionen mit 15*MA* bzw. 25*MA* führte bis zum Februar zu einer Verminderung des Mischsuspensionswertes gegenüber dem Mittelwert aus den Einzelsuspensionen, bis schließlich im März besonders bei der Mischung 5*C*/25*TA* ein Umschlag erfolgte (Reihe b). Die Annahme der Wirkung eines hemmenden Hormons *vor* dem Versuch (z. B. durch Inaktivierung von Fermenten im Abdomen) ist im Gegensatz zur Annahme der Wirkung eines den Sauerstoffverbrauch fördernden Hormons (z. B. durch Ansammlung von überschüssigen Fermenten in einem Körperteil) nicht möglich, es sei denn, man führte komplizierteste Hilfshypothesen an. Ebenfalls versagt eine Begründung der Hemmung durch die Abwesenheit eines aktivierenden Faktors (des C.-allata-Hormons). So bleibt aus logischen Gründen nur die Annahme eines *während* des Versuchs wirkenden Hemmfaktors, der in den Vorderteilen der schlafenden Tiere anzunehmen ist und dessen Wirkung sich in den Mischsuspensionen auch auf die „erwachten" Hinterenden erstreckt. Dieser Hemmfaktor (Diapausehormon der Unterschlundganglien ?) nimmt beim Aufwachen ab oder wird durch die Wirkung des C.-allata-Hormons verdeckt.

Die Reihe *c* zeigt, daß eine 5*MA*-Suspension eine ganz ähnliche Wirkung hat, weil sicherlich größere Mengen des Hemmfaktors im Blut kreisen. Da der Effekt der Hinterteile nicht nennenswert kleiner ist als der der Vorderenden, muß es fraglich erscheinen, ob der Hemmfaktor durch das Zermahlen einer innersekretorischen Drüse oder eines sezernierenden Gewebes wesentlich vermehrt wird. Beeinflußbar sind aber nur die Hinterenden der 15°– bzw. 25°– Tiere, kaum dagegen die Vorderteile, die nach Abb. 2 weniger „erwacht" sind bzw. weniger schlafen (Reihe d und e).

Es bleibt die Erklärung der Wirkung der *C*-Suspension auf die *TA*-Suspension, wenn beide aus gleich vorbehandelten, schlafenden Tieren gewonnen werden. Solche im März ausgeführten, allerdings nicht sehr zahlreichen Versuche haben zu einer Verminderung des Atmungswertes der Mischsuspension gegenüber dem theoretischen Mittelwert geführt (Reihe *a*, Spalte 8). Eine Deutung ist nicht einfach. Auffallend ist, daß die *C*-Suspension einen sehr hohen Atmungswert aufweist, während die Hinterenden (Thorax und Abdomen) zu dieser späten Jahreszeit den niedrigen Atmungswert beibehalten. Auch bei niedrigen Aufbewahrungstemperaturen findet zwar mit fortschreitender Jahreszeit hinsichtlich des Stoffwechsels ein gewisses „Erwachen" statt, doch beschränkt sich dies auf die Köpfe.

Nimmt man an, daß bei den tiefen Temperaturen der Hemmfaktor noch im Blut des ganzen Tieres vorhanden ist, so kann die vorgerückte Jahreszeit vielleicht doch eine Aktivierung der C. allata bewirkt haben. Das Hormon wird aber bei den tiefen Temperaturen nicht ausgeschüttet wie bei den hohen sondern gespeichert. In diesem Fall hat (wahrscheinlich im Gegensatz zu den entsprechenden

Versuchen mit erwachten Tieren) das Zermahlen einen Effekt (erhöhter Hormontiter). Es ist ferner zu berücksichtigen, daß in den *C*-Suspensionen etwa 3mal soviel Köpfe und damit Hormondrüsen enthalten sind wie in den *CP*-Suspensionen. Wenn nun in den Mischsuspensionen der Hemmfaktor aus den Hinterenden hinzukommt, so mag die aktivierende Wirkung des C.-allata-Hormons abgeschwächt werden (vgl. auch Reihe c, Spalte 6 u. 7). Es braucht nicht erwähnt zu werden, daß diese Erklärung nicht mehr als eine Arbeitshypothese sein kann.

Ergebnis. Alle Versuche lassen sich unter folgenden Annahmen erklären. Die Corpora allata werden beim Erwachen und wahrscheinlich auch bis zu einem gewissen Grade mit fortschreitender Jahreszeit aktiviert. Das Hormon wird dann aber bei tiefen Temperaturen nur gespeichert, im erwachten Zustand aber gleich ausgeschüttet. Die Wirkung des Hormons erstreckt sich vor allem auf die Hinterteile; hier resultiert eine Zellfermentvermehrung.

Die Stoffwechseldrosselung im Ruhezustand kommt durch ein hemmendes Hormon zustande, das wahrscheinlich im Blut kreist, somit also bei Vorder- und Hinterenden nachweisbar ist. Es wird vermutlich nicht gespeichert. (Oder es wird sowohl im Vorder- als auch Hinterende produziert, z. B. im Unterschlundganglion und Bauchmark; dann kann diese Aussage nicht gemacht werden.) Mit dem Erwachen nimmt die Hormonproduktion ab oder (bzw. und) die Wirkung des Inkrets wird durch das C.-allata-Hormon überlagert.

Es ist selbstverständlich, daß die hier angewandte Versuchsmethode grundsätzlich nur indirekte Schlüsse gestattet, die durch Versuche mit anderen Methoden erhärtet werden müssen.

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RÉSULTATS D'IMPLANTATIONS DE "GLANDES VENTRALES" À *LOCUSTA MIGRATORIA* L.

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Abstract—Implantations of ventral glands into *Locusta migratoria* at the beginning of the fourth instar result in a shortening of that instar and a reduction of the length of the body. The length of the femur of the metathoracic legs and of the pterotheca is also reduced after the fourth moult. The reduction of body length, femur length, and pterotheca length can be explained quantitatively by the shortening of the assimilation period during the fourth instar. The fourth moult has thus been hastened without any modification of growth or differentiation. It thus appears that the implantation of active ventral glands has merely moved the fourth moult without disturbing the process of growth or differentiation.

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Locusta migratoria est un acridien dont le développement comporte 6 stades séparés par 5 mues. Dans les conditions d'élevage standard (27 degrés, populations denses et faible humidité) le premier stade dure 3 à 4 jours, les suivants durent un peu plus longtemps, le stade IV dure 6 à 7 jours et le stade V qui est le dernier stade larvaire en dure 10 à 11. La 4ème mue est donc la dernière mue larvaire, la 5ème mue s'accompagnant de la métamorphose surtout marquée par le développement des organes du vol.

	1ère	2e	3e	4e		5e mue
Stades	I	II	III	IV	V	VI = Imago
Durée	3	4	6	6	10	70 Jours

FIG. 1. Schéma indiquant la chronologie du développement de *Locusta migratoria*.

Dans un travail antérieur (STRISCH-HALBWACHS, 1954) l'un de nous a pu montrer que des implantations de glandes ventrales pratiquées au début du stade V le raccourcissaient, donc avançaient la 5ème mue ou mue de métamorphose d'une manière indiscutable. Au contraire des implantations pratiquées au début du stade IV n'avaient que peu d'effet sur la durée de celui-ci et l'on pouvait se demander si les mues larvaires étaient elles aussi commandées par la glande ventrale.

Une technique améliorée nous a permis de reprendre cette question et d'obtenir cette fois un résultat exempt d'incertitude. Les animaux destinés à recevoir une greffe étaient prélevés chaque jour dans un élevage important au moment de leur 3ème mue et groupés dans de petites cages de manière à maintenir chez eux un grégarisme intense. Chaque lot était partagé en deux: la moitié des individus recevant une implantation de glandes ventrales, l'autre moitié élevée parallèlement servant de témoins. Les glandes ventrales ont été prélevées sur des larves du

stade V, 6 jours après leur 4ème mue. Le prélèvement a été exécuté par dissection sous Ringer de la tête du donneur. Deux paires de glandes ont été administrées à chaque animal récepteur par injection intra-abdominale au moyen d'une aiguille creuse de verre montée sur une seringue à injections hypodermiques. La greffe était pratiquée dans les 24 heures suivant la 3ème mue du récepteur donc au début de son stade IV.

Résultats. Ces implantations se sont montrées très actives; elles ont agi sur la durée du stade IV, sur la croissance au cours de ce stade et sur le développement relatif des ptérothèques.

Action sur la durée du stade IV. Le stade IV dure normalement 6 à 7 jours dans les conditions envisagées; comme le montre le tableau ci-dessous l'implantation de glandes ventrales le raccourcit notablement l'écart entre les témoins et les opérés est en moyenne de un jour et demi et il est égal à 5 fois son écart type donc incontestablement significatif.

Durée St. IV en jours	$\overline{X}$ = valeur moyenne de X	écart type de X	écart type de $\overline{X}$	t.σ	Nombre d'individus
Témoins	6,68	0,43	0,11		15
Opérés	5,18	1,12	0,29		15
Différence	1,50		0,31	0,64	

On peut donc conclure que la 4ème mue peut être avancée par des implantations de glandes ventrales aussi bien que la 5ème. Il ne s'agit donc pas d'une action précipitant la métamorphose mais provoquant une mue anticipée.

Action sur la croissance. Au cours de ce stade IV légèrement écourté l'animal accomplit une croissance moindre qu'au cours d'un stade IV normal.

Cette croissance a été appréciée de deux manières différentes: par la mesure de la longueur totale du corps (mesure rendue difficile par l'extensibilité de l'abdomen) et par la mesure du 3ème fémur dont la croissance est pratiquement homotétique à celle de l'ensemble du corps (JOLY, 1956). Ces deux types de mesures ont fourni des résultats tout à fait analogues.

Longueur totale	$\overline{X}$ = valeur moyenne de X	écart type de X	écart type de $\overline{X}$	t.σ	nombre d'individus
Témoins	31,3	1,25	0,39		10
Opérés	28,9	1,39	0,40		10
Différence	2,3		0,59	1,24	
Longueur Fémur III					
Témoins	16,00	0,37	0,12		10
Opérés	15,25	0,52	0,17		9
Différence	0,75		0,21	0,44	

On voit que dans l'un et l'autre cas la différence entre les animaux opérés et leurs témoins est d'environ quatre fois son écart type; cette différence est donc bien significative.

On peut se demander si cette réduction de taille n'est pas due simplement à la réduction de la durée du stade IV. On sait que chez la plupart des Insectes et nous avons vérifié qu'il en est bien ainsi chez *Locusta*, la croissance linéaire en fonction du temps est pratiquement une droite, du moins pour la partie moyenne du développement.

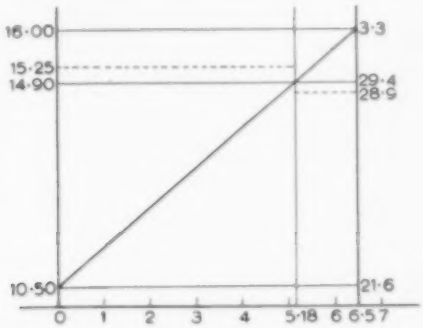

FIG. 2. Croissance du 3ème fémur et de la longueur totale du corps chez *Locusta migratoria*. Abscisses, temps en jours, ordonnées (à gauche) longueur du fémur en millimètres, à droite longueur totale du corps en millimètres (les origines et l'unité de longueur ont été choisies de manière à ce que la même ordonnée représente une longueur de 10,5 sur l'échelle des fémurs et de 21,6 sur celle du corps et que l'ordonnée correspondant à 16 sur l'échelle des fémurs représente 31,3 sur celle du corps).

Si donc nous traçons un graphique avec en abscisses les temps et en ordonnées la longueur du 3ème fémur, on sait que chez les témoins au point d'abscisse O (au moment de la 3ème mue) la longueur du 3ème fémur est 10,5 et au point d'abscisse 6,5 (moment de la 4ème mue) elle est de 16. Si nous joignons ces deux points par une droite celle-ci représente la croissance virtuelle du fémur pendant le stade IV. Si le stade IV est interrompu à 5,18 jours on voit qu'on peut s'attendre à un fémur de 14,9 mm, celui de nos opérés est de 15,25 ce qui est assez voisin.

Le même graphique peut être fait pour la longueur totale, il montre que à 5,18 jours on doit s'attendre à une longueur de 29,4 mm alors que nos opérés en mesurent 28,9 ce qui est voisin. Sur la figure 2 les deux graphiques ont été réunis en un seul en choisissant convenablement l'origine et l'unité de longueur pour les ordonnées du second graphique. On voit que les deux mesures tombent au voisinage du point théorique représentant la croissance à 5,18 jours. Les deux écarts étant assez faibles ne justifient aucune explication particulière, rien ne s'oppose donc à ce que l'on considère que la réduction de taille observée chez les animaux ayant subi une implantation de glandes ventrales est due au raccourcissement de la période d'assimilation qui sépare la 3ème mue de la 4ème.

Action sur les ptérothèques. Chez les Acridiens, les ptérothèques présentent une série de transformations au cours des dernières mues. Dans le cas de *Locusta* il y a retournement des ptérothèques à la 3ème mue, accroissement considérable à la 4ème et transformation en ailes à la 5ème.

La mue sur laquelle nous avons travaillé, la 4ème se signale donc par un grand accroissement des ptérothèques. Les chiffres cidessous montrent que l'implantation de glandes ventrales a une action très énergique sur la longueur des ptérothèques.

Longueur des ptérothèques	$\overline{X}$ = valeur moyenne de X	écart type de X	écart type de $\overline{X}$	t.σ	nombre d'individus
Témoins	10,50	0,58	0,19		10
Opérés	8,51	0,87	0,27		10
Différence	1,99		0,33	0,69	

Non seulement le raccourcissement absolu des ptérothèques (supérieur à 6 fois son écart type) est manifeste mais aussi leur raccourcissement relatif (leur longueur étant rapportée à celle du fémur). On peut donc se demander si l'explication fournie pour la longueur totale et pour celle du fémur est encore valable ici.

Il faut d'abord remarquer qu'étant donné le forte allométrie majorante présentée par les organes du vol il est impossible d'admettre pour leur croissance en fonction du temps une loi rectiligne comme nous l'avons fait pour le fémur. Pour déterminer au moins approximativement cette loi de croissance nous avons repris les

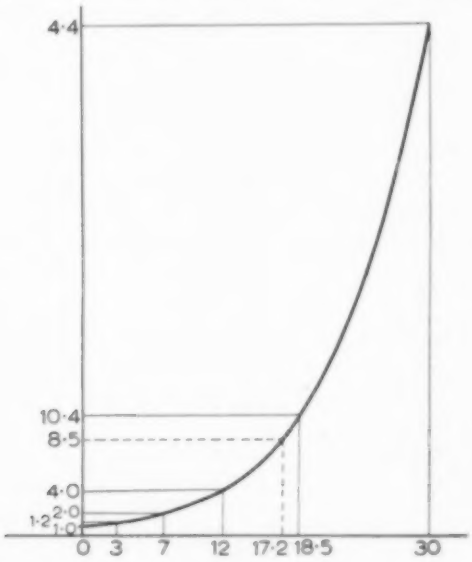

FIG. 3. Croissance des ptérothèques chez *Locusta migratoria.* Abscisses = temps en jours. Ordonnées = longueur des ptérothèques en mm d'après P. JOLY.

chiffres fournis par l'un de nous en 1956 pour la souche de *Locusta* sur laquelle nous avons travaillé. Ces chiffres nous ont fourni la longueur des ptérothèques à la naissance et après chacune des 5 mues. En joignant les 6 points ainsi obtenus par une courbe continue (Fig. 3) on obtient la courbe de croissance virtuelle des ptérothèques.

Sur cette courbe on constate que les ptérothèques 5,18 jours après la 3ème mue doivent mesurer 8,50 mm, or nos animaux greffés (qui ont subi leur 4ème mue 5,18 jours après la 3ème) ont des ptérothèques mesurant 8,51 mm. L'accord est donc parfait entre le nombre attendu et celui que nous avons trouvé. Il n'y a donc eu apparemment aucun changement dans la croissance des organes du vol mais seulement interruption de leur développement par la 4ème mue 1,5 jours plus tôt que normalement.

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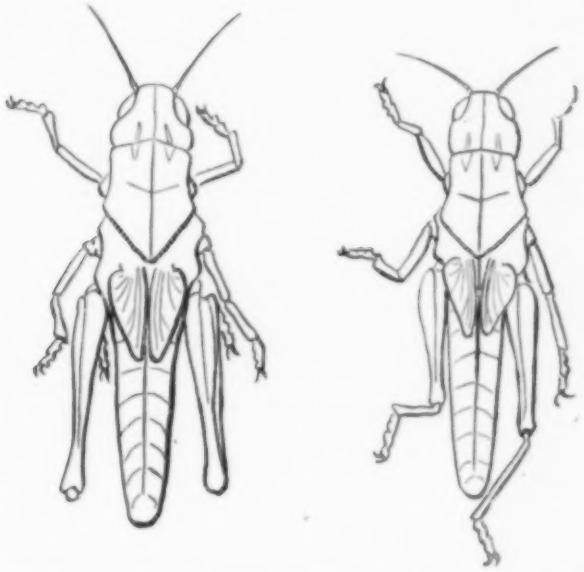

FIG. 4. *Locusta migratoria* stade V normal à gauche et obtenu par greffe de glande ventrale au stade IV à droite.

Discussion. On voit que les résultats obtenus à la suite de greffes de glandes ventrales au début du stade IV chez *Locusta migratoria* sont parfaitement cohérents.

La durée du stade IV se trouve raccourcie de 1,5 jours, soit environ 1/5 de sa valeur. La longueur totale de l'animal, celle de ses fémurs et celle de ses ptérothèques sont également diminuées et bien que fémurs et ptérothèques aient des lois de croissance extrêmement différentes la diminution qu'ils présentent par rapport aux témoins est justement celle que l'on pouvait attendre de la réduction observée sur la durée du stade IV.

Autrement dit la greffe semble n'avoir rien changé ni à la vitesse de croissance de l'ensemble du corps ni à celle du fémur ni à celle des ptérothèques mais elle a simplement avancé la 4ème mue qui survient ainsi plus précocement dans le développement du corps du fémur et des ptérothèques.

Cette interprétation est en parfait accord avec la notion d'un développement continu des Arthropodes (familière aux biométriciens) masqué par la présence de la cuticule et périodiquement extériorisé par des mues dont les positions par rapport au développement sous-jacent sont souvent assez variables.

Elle est aussi en bon accord avec l'observation que nous avons faite à plusieurs reprises d'animaux exceptionnels présentant un stade larvaire supplémentaire IV bis intercallé entre le stade IV normal et le stade V. Ces animaux qui morphologiquement rapellent beaucoup nos opérés présentent une morphologie et une croissance parfaitement normales, mais cette dernière au lieu d'être découpée en 6 stades par 5 mues l'est en 7 par 6 mues.

Par contre cette interprétation nécessite une révision de la notion actuellement classique d'hormone de croissance et différentiation identifiée par la majorité des auteurs avec l'hormone sécrétée par la glande ventrale ou prothoracique. Nos observations montrent que chez *Locusta migratoria* la glande ventrale sécrète une substance induisant la mue mais sans aucune action ni sur la croissance ni sur la différenciation.

SUMMARY

Two pairs of ventral glands, taken from larvae of the fifth instar on the sixth day after their fourth moult have been implanted into 15 *Locusta migratoria* larvae 24 hours after their third moult. This implantation has resulted in a shortening of the fourth instar and a reduction of the growth at the fourth moult:

	Duration 4th instar	Body length	3rd fem. length	Pterotheca length
Implanted animals	5·18	28·9	15·25	8·51
Controls	6·68	31·3	16·00	10·50

If it may be supposed that the growth during the moult is nearly proportional (at least for the organs with isometrical growth) to the time of the assimilation period preceding it, and the size of the implanted animals and that of their femora are exactly what one could expect after a fourth instar of 5·18 days instead of 6·68 days. The change in the length of the pterotheca is similar when allowance is made for their great allometry.

It thus appears that the implantation of active ventral glands has merely moved the fourth moult without disturbing the process of growth or of differentiation.

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DEHYDROGENASE ACTIVITY IN THE GUT AND ASSOCIATED TISSUES OF THE LARVA OF THE BLOWFLY *CALLIPHORA ERYTHROCEPHALA* MEIG.

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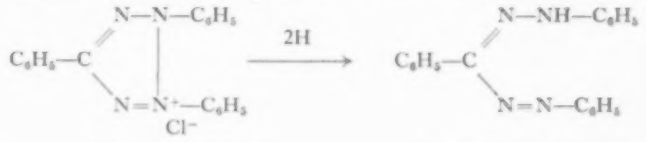
Abstract—Dehydrogenase activity in the gut and associated tissues of the larval blowfly, *Calliphora erythrocephala*, was readily demonstrated by using tetrazolium salts as hydrogen acceptors. Reduction of these salts to the corresponding insoluble and coloured formazan compounds was rapid at 20°–25° C. Of four tetrazolium salts used 2 : 5 diphenyl–3–(4–styrylphenyl)–tetrazolium chloride proved most satisfactory, the formazan crystals being readily observed in formal fixed tissues mounted in glycerol jelly. Dehydrogenase activity was detected in the salivary glands, midgut, digestive caeca, Malpighian tubes, and hindgut. Iodoacetate was a very effective inhibitor of the dehydrogenases present but malonate had no such effect. Several substances were tested as possible natural substrates; reduction of tetrazolium salt was effected in succinate and citrate solutions but not in solutions containing each of the following: fumarate, hippurate, lactate, malate, maleate, pyruvate.

INTRODUCTION

BIOLOGICAL oxidations *in vivo* generally involve the dehydrogenation of particular substrates by specific enzymes provided there are suitable substances available to act as hydrogen acceptors, *e.g.* oxidized cytochrome and various co-enzymes. *In vitro* studies on biological dehydrogenation have been extensive ever since methylene blue was introduced as a hydrogen acceptor by THUNBERG (1917). This technique involving the use of tissue breis or merely breis of small whole animals (AGRELL, 1947) does not facilitate the precise localization of dehydrogenase activity. From this point of view the use of tetrazolium salts is a distinct advance because tissue sections and tissue fragments can be used to study the dehydrogenases present.

Tetrazolium salts are amongst the relatively few kinds of organic compounds which are coloured in the reduced state. Reduction of tetrazolium salts leads to the formation of the corresponding water-insoluble formazan compounds.



Colourless T.P.T.Cl. Red triphenyl formazan

Amongst the commoner tetrazolium salts that have been used for the study of tissue dehydrogenases are tri-phenyl tetrazolium bromide (T.P.T.Br.), neo-tetrazolium (2 : 2 *p*-diphenylene-bis-3 : 5-diphenyl tetrazolium chloride), and Blue

tetrazolium, a dimethoxy derivative of neo-tetrazolium. Another salt, 2 : 5 diphenyl-3-(4-styrylphenyl) tetrazolium chloride (D.S.T.Cl.), has only recently become available* and as yet there is no published information on its use in histochemical work, but some of its properties, which will be subsequently demonstrated, indicate that it might be very useful indeed.

Most of the histochemical studies using tetrazolium salts to detect dehydrogenase activity have been carried out on mammalian tissues. In general, the information obtained has substantiated information available from extract studies, but of course more details of localization on the tissue and cellular level have appeared. WATERHOUSE and STAY (1955) and WATERHOUSE (1955) have shown that the dehydrogenase properties of insect tissues can be studied histochemically by using tetrazolium salts.

METHODS

1. *The reaction medium*

The small size and thickness of tissue of the larval blowfly gut makes it possible to demonstrate the presence of dehydrogenase very easily by placing freshly dissected whole guts in a suitable reaction mixture. Five-day-old larvae (EVANS, 1956, for details of culturing) were transferred to dry sawdust for 24 hr before dissection and only those with empty crops used. It was found convenient to dissect out the gut and associated tissues in an "insect Ringer" solution of the following composition : NaCl, 7·0 g, KCl, 0·2 g, $CaCl_2$, 0·2 g, $NaHCO_3$, 0·04 g, H_2O made up to 1 litre. After washing quickly in 0·05 M phosphate buffer the tissues were transferred quickly into the reaction mixture which comprised 1 ml 0·05 M PO_4 at pH 6·5, 0·5 ml 0·1 M sodium succinate, 0·5 ml 0·1% tetrazolium salt solution. pH 6·5 was chosen because in such a slightly acid solution there is no possibility of self-reduction of tetrazolium salt which can take place under strongly alkaline conditions (JAMBOR, 1954). Preliminary observations showed that at pH 6·5 reduction of tetrazolium salts by tissue dehydrogenases was adequate. It was not so quick as at pH 8·0, but on the other hand at pH 5·3 there was very reduced activity. Sodium succinate was usually added to the reaction medium, although it soon became evident that there was sufficient endogenous substrate present to enable the reduction of tetrazolium salt to proceed without addition of substrate. The presence of 0·1 M sodium succinate in the reaction mixture reduced to a minimum the possibility of failure to detect dehydrogenase activity because of local low substrate concentrations.

JAMBOR (1954) reported that T.P.T.Cl. reduction was markedly influenced by light. Indeed he stated that in darkness enzymatic reduction (using a yeast suspension) and ascorbic acid reduction did not take place. Experiments using larval blowfly gut and also cockroach leg muscle showed that for T.P.T.Cl. and D.S.T.Cl. incubation under diffuse daylight, electric light, or in darkness made no appreciable difference to the reaction. JAMBOR (1954) also stated that T.P.T.Cl. disappeared rapidly from solution in the light. Although there was no observable decrease in the coloration of tissues incubated with freshly prepared and laboratory stored solutions of T.P.T.Cl., solutions of the various tetrazolium salts were kept in the dark. Under these conditions and at room temperature 0·1% tetrazolium salt solution did not deteriorate over a period of 5–6 weeks.

2. *Treatment of stained tissue for histochemical and cytochemical observation*

Coloured tissues were transferred from the reaction mixture to formal-calcium (BAKER, 1944) which proved to be the most suitable fixative. Fixation was rapid, treatment for any

* Obtainable from May & Baker Ltd., Dagenham, England.

length of time between 5 and 30 min being satisfactory. Alcohol fixatives gave poor fixation of the tissue, whilst fixatives containing organic solvents were useless because of the solubility of formazan in them. The fixed tissues were mounted in a glycerol jelly medium. The usual permanent mounting media could not be used; Canada Balsam because the prior treatment in a dehydrating liquid dissolved out the formazan, and "Euparal" because of the solubility of formazan in it.

RESULTS

1. *Histochemical demonstration of dehydrogenase activity using 4 tetrazolium salts*

Larval gut tissues incubated in the various tetrazolium salt solutions all showed rapid coloration in regions of the midgut. Table 1 summarizes the observations made on glycerol-jelly preparations of tissues incubated in tetrazolium reaction

TABLE 1—COMPARISON OF THE PROPERTIES OF 4 TETRAZOLIUM SALTS AS INDICATORS OF DEHYDROGENASE ACTIVITY IN TISSUES INCUBATED AT 25° C

	T.P.T.Cl.	T.P.T.Br.	B.T.	D.S.T.Cl.
Appearance of colour	20 min	20 min	20 min	7 min
Nature of colour in most intense regions	Pink	Orange-pink	Violet	Deep red
Salivary glands	Formazan granules in cytoplasm	Formazan granules in cytoplasm	Formazan granules in cytoplasm	Formazan granules followed by crystals in cytoplasm
Midgut	Formazan granules in some regions	Formazan granules in some regions	Formazan granules in some regions	Large and small formazan crystals widely distributed
Malpighian tubes	Formazan granules	Formazan granules	Well differentiated formazan granules	Formazan crystals
Hindgut	Formazan granules in some regions	Formazan granules in some regions	Formazan granules in some regions	Formazan crystals

mixtures for 1 hr. The Malpighian tubes, because of their small dimensions, proved to be very useful for the rapid examination of the formazan crystals which formed. Their examination soon showed that D.S.T.Cl. gave the most consistent and marked reaction. The D.S.T.Cl. formazan crystals found in the Malpighian tubes, salivary glands, and crop were usually short and needle-like, but in the other regions of the gut they were often much larger and tended to grow together (see plate Fig. 1).

2. Dehydrogenase activity using D.S.T.Cl.

The first change to be observed in larval gut tissues incubated in a D.S.T.Cl. reaction mixture was an intense red coloration in the anterior midgut region where there was a differential coloration with groups of cells, seemingly randomly distributed, appearing more deeply stained (see plate Fig. 2). However, after further incubation, 30–60 min, this anterior midgut differential staining effect was replaced by a more generalized staining and at the same time the pattern of staining along the whole gut was established. Immediate examination of glycerol-jelly mounts of 1 hr incubated guts shows little or no trace of formazan crystals but they appear in 2–3 hr, and after 24 hr the pattern of their distribution is readily ascertained.

The cells of the larval salivary glands show, at first, the characteristic appearance of a central non-stained nuclear area surrounded by a dark granular cytoplasmic area which is itself surrounded by a clear cytoplasmic area beneath the cell membrane. The formation of crystals usually obliterates the details of the intracellular staining pattern in the salivary gland cells.

The larval proventriculus was stained a light blue-violet colour and the formazan crystals when formed were distributed generally throughout the tissue.

The larval midgut can be conveniently divided into 3 regions, anterior midgut, mid-midgut, and posterior midgut (WATERHOUSE and STAY, 1955). Table 2 summarizes the observations made on the colour pattern and formazan crystal formation in the midgut. The anterior zone of the anterior midgut was short,

TABLE 2—COLORATION AND FORMAZAN DISTRIBUTION IN *Calliphora* MIDGUT AFTER D.S.T.Cl. TREATMENT

Region	Coloration and crystal distribution
Ant. midgut	
Ant. zone	Light blue coloration. Very few crystals.
Post. zone	Heavy red coloration. Dense crystals.
Mid-midgut	Generally very light staining but some cells heavily stained; very few crystals apart from localized cells.
Posterior midgut	Red coloration but not so heavy as in ant. midgut. Tendency for bands of heavier coloration along gut. Crystal formation dense and fairly evenly distributed.

approximately equal in length to the proventriculus. Plate figs. 1 and 2 illustrate the appearance of formazan crystals in various regions of the midgut.

The larval digestive caeca often showed a differential staining pattern, some cells being stained a light blue-violet whilst others were not stained at all. Crystal formation was also variable, formazan often being present in some cells but not in others.

The Malpighian tubes usually stained deep blue-violet and formazan crystal formation was marked, the cells containing large numbers of small needle-like

crystals (see plate Fig. 3). Some regions of the Malpighian tubes did not show any reaction, there being little uniformity in the pattern of staining in different animals.

The most characteristic feature of the hindgut reaction was the differential staining effect. Here a longitudinal band extended from the anterior hindgut to the posterior end. Whilst formazan crystals appeared in all regions of the hindgut they were present in greatest concentration along the colour band (plate Fig. 4).

3. *Inhibition of larval gut dehydrogenases*

Addition of substances, known to be inhibitors of mammalian dehydrogenases, to give a reaction mixture concentration of 0·016 M showed that sodium iodoacetate was a very effective inhibitor, there being no trace of colour or of formazan crystals. Potassium ferricyanide addition appeared to diminish the intensity of the colour reaction, whilst the addition of malonate and sulfanilic acid did not produce any visible inhibitory effect. These observations were confirmed when guts previously frozen were incubated in reaction mixtures containing succinate.

4. *Substrates for larval gut dehydrogenases*

The ability of larval gut tissues to reduce tetrazolium salts in the absence of added substrate precludes deductions being made of the utilization, as a substrate, of any added substance. However, the well-established observation that prior freezing of tissues inhibits endogenous dehydrogenase activity enables tests to be made. The gut tissues from 6-day-old larvae were frozen for 24 hr in 0·05 M phosphate buffer at pH 6·5 at a temperature just below 0° C. After thawing the guts were transferred to reaction mixtures containing phosphate buffer at pH 6·5, D.S.T.Cl., and a substrate solution. Duplicate reaction mixtures to which cyanide was added were also set up. The results obtained after 2 hr incubation at 25° C indicate that of the substrates used, and both in the presence and absence of cyanide, only succinate and citrate showed positive reactions, i.e. the typical coloration and subsequent formazan crystal formation. The addition of the following substances resulted in negative reactions in the presence and absence of cyanide: fumarate, hippurate, lactate, malate, maleate, pyruvate.

DISCUSSION

The differential colouring effects of the midgut are probably correlated with the differential distribution of lipid material. Zweifach *et al.* (1951) and Shelton and Schneider (1952) have demonstrated that formazan is soluble in cellular lipid material and hence the characteristic coloration of lipid rich larval gut tissue when incubated in tetrazolium solutions. The possibility that tetrazolium salt is reduced by lipid droplets must not be ignored (Shelton and Schneider, 1952), but incubation of a slightly acid D.S.T.Cl. solution and olive-oil suspension under bacteria-free conditions, without any observed change in colour over a period of days suggests that this is unlikely. The appearance of formazan crystals or granules

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FIG. 1. Part of anterior midgut (× 200 approx.) showing large formazan crystals.

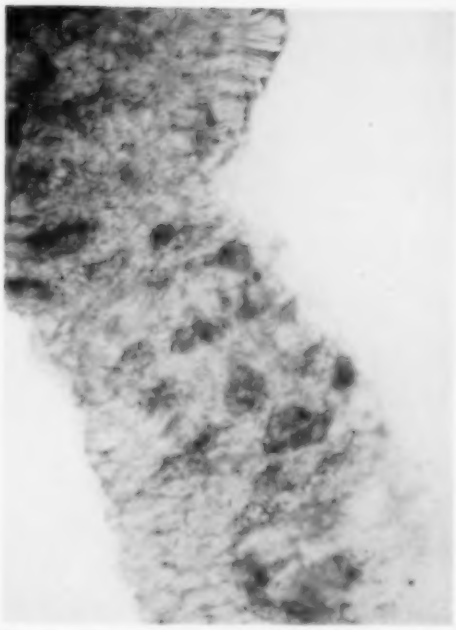

FIG. 2. Part of larval mid-midgut (× 80 approx.) showing localized regions containing abundant formazan crystals.

FIG. 3. Part of Malpighian tube (× 240 approx.).

FIG. 4. Part of hindgut (× 60 approx.) showing differential staining and formazan distribution along the length of this region.

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in the midgut after the coloration effect indicates that the lipid becomes saturated with formazan before crystallization takes place.

The granular appearance of the formazan formed in T.P.T.Cl., T.P.T.Br., and B.T. solutions was quite distinctive in the salivary glands and Malpighian tubes, but in the midgut and hindgut their presence tended to be masked because of the thickness of the gut tissues in these regions. On the other hand, the formazan of D.S.T.Cl. gave characteristic needle-like crystals similar to tetrazolium violet formazan (SHELTON and SCHNEIDER, 1952). Their presence in midgut and hindgut was easy to see, particularly in the anterior midgut where the formazan crystals were relatively large in size. Although the crystal size of D.S.T.Cl. formazan is large and of little use for the precise intracellular location of dehydrogenase activity D.S.T.Cl. is extremely useful as a rapid means of detecting dehydrogenase activity in small tissues or tissue fragments under different experimental conditions.

The localization of formazan crystals to certain cells or groups of cells in the mid-midgut region suggests that a differential pattern of intense dehydrogenase activity is probably general for this region of the gut of blowfly larvae because similar results have been recorded in *Lucilia cuprina* by WATERHOUSE and STAY (1955). The more intense coloration along a longitudinal band in the hindgut is also similar to that recorded for *Lucilia cuprina* by WATERHOUSE (1955), but it is clear that this is a region of greater relative activity only since formazan crystals were abundant in other regions of the hindgut as well. WATERHOUSE (1955) reported that dehydrogenase activity was confined to the longitudinal band of cells in the central region of the hindgut only, there being a short region just behind the position of entry of the Malpighian tubes into the gut and a short region just before the anus showing a negative reaction. In *Calliphora* the longitudinal band extends in a similar central region of the hindgut but formazan crystals are present along the whole length of the hindgut, thus indicating general dehydrogenase activity in the hindgut cells but with a region, the longitudinal band, exhibiting intense activity.

The results of the tests carried out with inhibitors show some interesting features, particularly the non-inhibitory effect of malonate. Malonate has been accepted as a specific inhibitor of mammalian succinic dehydrogenase for many years, although PARDEE and POTTER (1949) have more recently established that malonate can inhibit at least one other oxidizing enzyme, oxalacetate dehydrogenase, in rat tissues. Histochemical observations on mammalian tissues confirm the biochemical conclusions concerning malonate as an inhibitor of succinic dehydrogenase (SELIGMAN and RUTENBERG, 1951; PEARSON and DEFENDI, 1954). WATERHOUSE and STAY (1955) and WATERHOUSE (1955) infer from qualitative tests that 0·1 M malonate partially inhibits dehydrogenase activity in the midgut and hindgut of *Lucilia cuprina*. In view of these results and those of the *Calliphora* experiments it would be very desirable to have quantitative data on the inhibitory effect of malonate.

Malonate inhibition of mammalian succinic dehydrogenases is explained by competitive inhibition; the malonate molecule combines with active groups of the

enzyme instead of the structurally similar succinate molecule. It is difficult to understand why blowfly succinic dehydrogenase is not inhibited for precisely the same reason. It appears that there must be some fundamental difference in molecular structure and properties between mammalian and blowfly succinic dehydrogenase.

The effectiveness of monoiodoacetate as a *Calliphora* succinic dehydrogenase inhibitor was also demonstrated for *Lucilia cuprina* by WATERHOUSE and STAY. This property also contrasts with the properties of mammalian succinic dehydrogenase for which iodoacetate acts as an inhibitor only when present in high concentration (BALDWIN, 1953). Iodoacetate inhibition is accounted for by the iodoacetate molecule reacting with —SH groups of the enzyme, upon the presence of which the activity of the enzyme depends. The presence of oxidizing agents usually inhibits mammalian succinic dehydrogenase activity because adjacent pairs of —SH groups are oxidized to form —S—S—linkages. However, as far as *Calliphora* succinic dehydrogenase is concerned, the addition of the oxidizing agents, ferricyanide and sulphanilic acid, does not produce this effect, at least to a marked degree.

Prior freezing of *Calliphora* larval gut tissues followed by incubation with different possible substrate substances indicates that succinate and citrate undergo dehydrogenation. Succinate is probably a natural substrate for a larval gut dehydrogenase, and citrate is probably utilized by being converted to iso-citrate before dehydrogenation. In *Lucilia cuprina* the midgut and hindgut oxidized succinate but citrate is not oxidized. The inability of *Lucilia cuprina* gut to oxidize malate and fumarate points to a similarity with *Calliphora*. AGRELL (1947), using a methylene-blue technique, stated that dehydrogenase present in *Calliphora* pupal breis could utilize a very wide range of substrates. Amongst these were fumarate, lactate, and pyruvate which are not substrates for *Calliphora* larval gut dehydrogenases. It appears that, apart from succinic dehydrogenase, the pupal dehydrogenases must come from larval tissues other than the histolysing gut or they are synthesized *de novo* during metamorphosis.

From the qualitative observations made on gut tissue it can be inferred that the addition of cyanide does not markedly influence the reduction of tetrazolium salt. Cyanide is a very effective inhibitor of cytochrome oxidase, and so it would be expected that if a cytochrome *c*-cytochrome oxidase system were present then formazan production would be greater in the presence of cyanide because of the absence of competition between tetrazolium salt and oxidized cytochrome *C* to act as hydrogen acceptor. It is likely therefore that cytochrome oxidase is not involved in the oxidation mechanisms in gut tissue. For *Cecropia* larvae PAPPENHEIMER and WILLIAMS (1954) have suggested that in the midgut autoxidizable and cyanide-resistant cytochrome b_5 transfers electrons to atmospheric oxygen. If such a system operates in *Calliphora* larval gut tissue, then the absence of any effect of cyanide addition on tetrazolium salt reduction can be accounted for.

SUMMARY

1. The use of tetrazolium salts affords a rapid means of demonstrating dehydrogenase activity in insect tissues under a variety of experimental conditions.

2. Of four tetrazolium salts investigated, 2 : 5 diphenyl-3-(4-styrylphenyl)-tetrazolium chloride gave the most satisfactory histochemical results, but its value for cytochemical work is limited.

3. Dehydrogenase activity was demonstrated in the following tissues from 6-day-old larvae: salivary glands, proventriculus, anterior midgut and digestive caeca, mid-midgut, posterior midgut, Malpighian tubes and hindgut.

4. Iodoacetate was a very effective inhibitor of gut dehydrogenase, but malonate had no apparent inhibitory effect.

5. Of a range of test substances examined as possible natural substrates only succinate and citrate were capable of dehydrogenation.

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THE EUROPEAN CORN BORER, *PYRAUSTA NUBILALIS* (HÜBN.), AND ITS PRINCIPAL HOST PLANT—VI
HOST PLANT RESISTANCE TO LARVAL ESTABLISHMENT*

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Abstract—A study has been made of the role of growth-inhibiting chemical factors in the resistance of certain dent corn inbreds to the establishment and survival of European corn borer larvae. The inbred lines employed were WF9, W204, W210D, W22, and W22RB. Total resistance factor activity, as determined by bioassay, was found to be in good agreement with resistance ratings of the inbreds as determined in field tests by other workers. It was found that the whorl tissues on which borer larvae of the first generation feed may contain high levels of ether-soluble resistance factors, but in most cases only low levels of ether-insoluble resistance activity. The chemical factor primarily responsible for resistance to leaf feeding was found to be Resistance Factor A (6-methoxy-benzoxazolinone). At the growth stage in which borer attack on the tassel occurs, the tassels of all the inbreds displayed very low resistance activity, and little or no intervarietal resistance was detected. After the open tassel growth stage the internodes of W22 and W22RB and the leaf sheaths of W22RB showed good intervarietal resistance activity. Other tissues tested, including ear structures, did not display appreciable varietal differences in resistance factor activity.

The relative resistance of early plant growth stages compared to later growth stages was termed intravarietal resistance and some of its characteristics were determined. Resistance factor activity of whorl leaves of the borer zone was shown to decline as the tassel length increased. The activity in other borer-significant tissues did not necessarily decline as tassel length increased, however, and intravarietal resistance was not always at its maximum in the whorl stage of the different inbreds. It was shown that varietal differences in resistance factor activity were not explicable solely in terms of differences in resistance associated with the growth characteristics of the plant.

There appeared to be at least three chemicals contributing to the total resistance factor activity of the corn plant. Resistance Factor A was important in the resistance of early plant growth stages, but negligible after the development of a visible tassel. Resistance Factors B and C contributed about equally to the resistance factor activity found in internode, leaf-sheath, husks, and silk tissues. It was postulated that the resistance of corn to different parasitic organisms is caused by the same complex of chemicals responsible for resistance to the European corn borer.

THE establishment of newly hatched European corn borer larvae on a corn plant is a biological occurrence whose success depends on a precarious balance among a number of factors operating for or against larval survival. Successful establishment is accomplished largely by sheer force of numbers, for more than 75% of the larvae fail to survive their first 48 hr., and usually far less than 10% attain maturity (PAINTER and FICHT, 1925; CAESAR, 1926; SPRINGER, 1930; HUBER, 1936). The rapid increase in population and extension of geographical distribution displayed by this insect since its introduction into North America has undoubtedly

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been brought about by virtue of high host-plant density, rather than by the excellence of its adaptations to any of its numerous host-plant species. The high mortality among borer larvae is caused, in large part, by physical factors such as mechanical crushing by plant parts, drowning, dislodging, desiccation, predation, and exposure to ultra-violet irradiation (PAINTER and FICHT, 1925; SPRINGER, 1930; LADUIZHENSKAYA, 1936; STIRRETT, 1938). In addition, however, there are also plant-borne factors which contribute to larval mortality. Such plant factors offer a resistance to borer establishment, and it is their identity, distribution, biological significance, and agronomic importance that is the subject of the study herein reported.

Soon after the discovery of the European corn borer in North America it was a generally accepted observation that corn planted early in the growing season was more liable to damaging first-generation borer infestation than was that planted relatively late (CAFFREY, 1919; FELT, 1921). The larger, more mature plants were found not only to be more attractive to ovipositing borer moths, but larval establishment and survival on them was also more successful (CAFFREY and HUBER, 1928; HOUSER and HUBER, 1929; NEISWANDER and POLIVKA, 1930; FICHT, 1931, and many subsequent workers). These observations led a number of workers into more or less detailed explorations of the relationships between growth stages of the corn plant and the suitability of the plants as hosts for borer larvae. Based on the percentage of larvae surviving on plants infested at different developmental stages, the suitability of the corn plant as a borer host increases as the plant grows, and reaches a maximum at about the time the tassel emerges from the whorl (NEISWANDER and POLIVKA, 1930; BEARD and TURNER, 1942; PATCH, 1943; DICKE *et al.*, 1949; LUCKMAN and DECKER, 1952). Survival is from three to several times higher on plants infested after tasselling than on plants infested prior to the appearance of the tassel (DICKE and PENNY, 1954). A number of measurable plant-growth characteristics have been described as associated with the relative susceptibility of different-aged corn plants to borer establishment. Such characteristics include: plant height (MEYERS, 1930); total leaf area (FICHT, 1932); number of days from average silking (POLIVKA, 1931; MEYERS, 1929; SCHLOSBERG and BAKER, 1939); relative prominence of the tassel (NEISWANDER and POLIVKA, 1930); the tassel ratio, which is the height of the plant from the ground to the tip of the tassel or tassel primordium, divided by the height of the plant from the ground to the tip of the longest leaf and multiplied by 100 (LUCKMANN and DECKER, 1952); and tassel length, which is the distance from the apical node to the tip of the tassel or tassel bud (LUCKMANN, 1956). BATCHELDER (1946) devised a system of classifying the growth stages of corn into arbitrary categories based on the number of leaves present, the extent of development of the tassel, the presence and colour of silks, etc. Although such a system has the obvious limitations of any attempt to fit a continuum into a series of defined stages, it has proved useful in descriptive research on borer feeding on corn plants (BEARD and TURNER, 1942; BATCHELDER, 1949; DICKE and PENNY, 1954). The plant characteristics listed above as having been found to be associated with relative susceptibility are

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only descriptive in nature, and there is no evidence that borer establishment is causally dependent on any of them. Some of the correlated characteristics have proved to be agriculturally useful, however; this has been the case with tassel ratio and tassel length measurements.

The resistance to borer establishment which is correlated with differences in maturity among corn plants of identical genotype may be defined as ***intravarietal resistance***. The term intravarietal resistance is needed to distinguish this type of resistance from *intervarietal resistance*, which is the relative resistance among corn plants of identical growth stage but of different genetic composition. It has long been recognized that different varieties of corn differ in their resistance to borer establishment, and as early as 1928 it was expected that agronomists would soon produce genetic lines that would be highly resistant to the borer (CAFFREY and HUBER, 1928; HUBER and NEISWANDER, 1930). That borer resistance of corn plants is a heritable characteristic was demonstrated by MARSTON (1929). It is, of course, well known that genetic lines of corn differ in their growth characteristics, with some lines tasselling and silking in a shorter growing period than is required by others. The existence of intervarietal resistance, as defined above, to European corn borer establishment was, from the outset, questioned by many workers. It was found that slow-growing varieties were more resistant than those that displayed rapid growth (CUTRIGHT and HUBER, 1928; MEYERS, 1929, 1930; POLIVKA, 1931; FICHT, 1931, 1936; NEISWANDER and POLIVKA, 1930; and others). A 2–5% decrease in surviving borer population was found to be associated with each day's delay in the silking date displayed by different varieties (MEYERS, 1929, 1930, 1937; POLIVKA, 1931; NEISWANDER and POLIVKA, 1930). NEISWANDER and HUBER (1929) found that the time of tasselling was important to resistance, with early tasselling varieties being the most susceptible. They postulated that the growth pattern associated with earlier tassel development was more perfectly timed to early larval growth. Results and observations such as these have led a number of workers to the concept that varietal differences in resistance are the result of some varieties being in an unfavourable growth stage at a time which is critical to the establishment of the European corn borer (CUTRIGHT and HUBER, 1928; FICHT, 1931, 1936; HUBER, 1939; BEARD, 1943; TURNER and BEARD, 1950; BECK, 1951).

There is, however, considerable evidence that intervarietal resistance exists and can be distinguished from the intravarietal effects discussed above. Comparisons of larval establishment on different genetic lines of corn disclosed varietal differences that could not be accounted for in terms of differences in growth rates (MARSTON, 1930; HERR and HUBER, 1931; PATCH, 1937; SCHLOSBERG and BAKER, 1939). LUCKMANN and DECKER (1952) found that survival of first-generation borer larvae showed a high positive correlation with the tassel ratio of the host plant, but the regression of survival on tassel ratio was found to differ in slope for different genetic lines of corn. Until recently, plant resistance to borer establishment was considered to be operative against only the first-generation larvae feeding in the whorl portions of the plant, with no resistance being shown towards establishment of second generation (PATCH and DEAY, 1948; PATCH, 1943; EVERLY, 1946). The

finding that some genetic strains display resistance to second-generation establishment in leaf sheath and other tissues (DICKE and PENNY, 1954, 1956; DICKE and GUTHRIE, 1955) is indicative of important intervarietal resistance.

Newly hatched borer larvae which feed on the whorl tissue of young or resistant corn plants not only show a high incidence of mortality, but also the growth rate of the survivors is reduced (HUBER, 1938; PATCH, 1943; BECK and LILLY, 1949; DICKE and GUTHRIE, 1955; LUCKMANN, 1956). The identification of the factors responsible for these effects has been investigated by a number of workers. Several investigations have resulted in the postulation that the tissues of resistant plants fail to meet the nutritional requirements of the young larvae (HOUSER and HUBER, 1929; BOTTGER, 1937; HUBER, 1938; BECK and LILLY, 1949; DICKE and PENNY, 1954; LUCKMANN, 1956). Research on this aspect of host-plant resistance was seriously impaired, however, by the lack of experimental methods for culturing borer larvae apart from their host, and by the absence of any knowledge of the insects' nutritional and feeding requirements. BOTTGER (1942) devised an artificial substrate on which borer larvae would feed. Using BOTTGER's substrate, BECK *et al.* (1949) demonstrated some of the borer's nutritional requirements. The development of aseptic culturing methods (BECK and STAUFFER, 1950) and further information on the larval nutritional requirements (BECK, 1950, 1953) has made possible an experimental approach to the problem of the rôle of nutrition in host-plant resistance (BECK, 1956d). It was found that the manifestations of resistance were not consistent with the manifestations of nutritional deficiencies, and the conclusion was drawn that host-plant resistance is not caused by nutritional deficiencies in the corn plant.

The high mortality and low growth rate of young borers feeding on resistant plants is also indicative of a failure of the plant to provide the attractants and feeding stimulants required for normal larval feeding. Whorl leaves of susceptible plants show more numerous and larger feeding punctures per infesting larva than do those of resistant plants (HUBER, 1937; DICKE and PENNY, 1954). Experimental studies of the feeding behaviour of first instar larvae under controlled laboratory conditions have shown: (1) that borer feeding is not dependent on plant-borne feeding stimulants (BECK, 1956a); (2) that different parts of the corn plant are equally attractive to the borer larvae (BECK, 1956b); and (3) that young corn plants do not contain feeding deterrents which would account for their resistance to borer establishment (BECK, 1956d). It has been found that sugars have a marked influence on the feeding behaviour of borer larvae (BECK, 1956a, c) and on their distribution on the plant (BECK, 1956d). Varietal differences in resistance could not be accounted for on this basis, however (BECK, 1957).

The manifestations of host-plant resistance—high larval mortality and poor larval growth—are also consistent with the idea that the resistant plant tissues contain toxic or growth-inhibiting chemicals. Adding corn plant extracts to purified borer diets and maintaining the cultures under aseptic conditions resulted in the detection of such substances (BECK, 1951). Detailed investigations have disclosed the presence of at least two borer-growth inhibitors in the tissues of

young corn plants (BECK and STAUFFER, 1957). The concentration of one of them (termed Resistance Factor A) was found to be high in young corn plants, but to decline rapidly as the plants grew more mature; varietal differences in concentrations were also found (BECK and STAUFFER, 1957; LOOMIS *et al.*, 1957). Resistance Factor A was isolated (LOOMIS, 1956), characterized as 6-methoxy-benzoxazolinone, and synthesized (SMISSMAN *et al.*, 1956). It is quite apparent that corn-plant resistance to the establishment and survival of European corn borer larvae is caused, at least in major part, by the presence of chemicals in the host-plant tissues which have a toxic action against the young borer larvae.

The experimental results presented and discussed below were obtained in a study of the chemical factors involved in the resistance displayed by a selected group of dent corn inbreds towards the establishment of both first- and second-generation European corn borer.

METHODS AND MATERIALS

The corn plants used in this study were dent corn inbreds of lines WF9, W204, W210D, W22, and W22RB. These genetic lines were chosen on the basis of their relative resistance to first-generation borer-leaf feeding according to USDA field rating reports received by the Wisconsin Agricultural Experiment Station. The inbreds are listed above in order of increasing rated resistance to leaf feeding, with WF9 being the most susceptible and W22 and W22RB the most resistant. The inbreds were drill planted in row plots. Three randomly distributed replicate plots were used for each line. Fertilization and cultivation procedures normal to University Farm practices were maintained. All plots were seeded on 19 May 1956, and this planting was consistent with agricultural practice in the area.

When the corn plants reached an average extended height (distance from ground to the tip of the longest leaf) of about 33 inches, weekly measurements of growth were begun. Ten plants were chosen at random from each plot each week for determination of extended height, tassel height (distance from ground to tip of tassel or tassel primordium) tassel ratio

$$\frac{\text{Tassel height}}{\text{Extended height}} \times 100,$$

and tassel length (length of tassel from tip to apical node). Plant samples were also taken periodically for bioassay and chemical analysis. For these samples, from 30 to 40 plants were collected from each plot, and the growth measurements described above were taken. The plants were then dissected and samples of 400 to 600 g of the tissues to be analysed were weighed out, placed in polyethylene plastic bags, and quick frozen. The samples were maintained in a frozen state until they were subjected to further processing (a matter of 1 to 7 days).

Concentrates were prepared for bioassay of resistance factors from the frozen tissue samples. The plant tissue was dried for 18 hours at 40° C in a low-pressure drying oven. The dried sample was weighed, allowing calculation of per cent moisture in the fresh tissue. The sample was then ground to a powder in a motor-driven drug mill. The sample was then extracted thoroughly with boiling water; the residue was discarded, and the aqueous extract was concentrated under reduced pressure until one millilitre of extract represented 5 grammes of fresh plant tissue. A portion of the aqueous concentrate was acidified and extracted with diethyl ether. This extraction was carried out in a separation funnel, using 12 successive small volumes of ether. The ether extracts were combined, evaporated to dryness, and the residue was taken up in water. The pH was adjusted to 6·5, and the volume

was made equal to the volume of crude concentrate extracted. The extracts so produced were used in bioassays to estimate the activity of *ether-soluble resistance factors*. After ether extraction, the aqueous plant concentrates were boiled to drive off any ether left in solution, the pH was adjusted to 6·5, and the preparations were centrifuged to remove an insoluble sludge. These extracts were used in bioassays to estimate the activity of *ether-insoluble resistance factors*. When these procedures were first used, it was thought that the only resistance factor present in the ether extract would be Resistance Factor A (6-methoxy-benzoxazolinone). It was subsequently found, however, that at least one other ether-soluble resistance factor was present in some of the tissue samples. The ether soluble extract activity reported under "Results" is not attributable, therefore, to the same chemical in every instance.

The organisms employed for bioassays were the European corn borer on purified diets, and *Penicillium chrysogenum* on honey-peptone agar media. Borer larvae were used in most of the early work on the resistance problem, but it has been shown (BECK and STAUFFER, 1957) that *Penicillium* and the borer display approximately the same sensitivity to the growth-inhibiting effects of the resistance factors. Because of their rapidity and statistical superiority, *Penicillium* assays have been used much more than the borer in the more recent work. The assay methods have been described in detail in previous papers of this series (BECK and STAUFFER, 1957; LOOMIS *et al.*, 1957). The *Penicillium* assay was standardized in the present study in that the plant extracts were added to the agar media in amounts such that one millilitre of medium contained the extract of 2 g of fresh plant tissue. Statistical analyses of the assay results were carried out on the original assay data, which were colony diameters of *Penicillium* and larval weights of the borers. Uninhibited controls were included in every assay and were used as the basis for calculation of per cent inhibition (%*I*). Statistical consideration of the resistance factor activity of the different plant samples involved comparing the results obtained with the different inbreds with those obtained from comparable WF9 samples, rather than with the uninhibited controls. Ether-soluble and ether-insoluble resistance activities were assayed separately and were expressed in terms of per cent inhibition. Where an expression of total activity was desired, an estimate was made by the simple addition of the per cent inhibition caused by the two fractions assayed separately. This method resulted in an apparent total activity greater than 100% inhibition in a few cases, but is suitable for comparative purposes. The conditions under which the bioassays were run were artificial in comparison to the conditions encountered in field aspects of the resistance problem. For this reason, it was considered likely that the per cent inhibition observed in the bioassays had only a relative relationship to actual borer survival and growth on corn plants. For the purpose of comparing the resistance factor activity found in comparable samples from different inbreds, resistance factor activity ratios were calculated. These ratios were obtained by dividing the per cent inhibition produced by a given inbred tissue sample by that produced by the comparable WF9 sample. This procedure involved the assumption that all the tissues of WF9 represented a typical susceptible condition, and allowed an estimation of intervarietal differences in resistance.

Some small plant tissues samples (1·0 to 2·0 g) were collected for use in the development of a quantitative chemical method for the estimation of 6-methoxy-benzoxazolinone (RFA) and for analysis of sugars present in the plant tissues. These samples were stored frozen until used, and then were extracted with diethyl ether for 3 hr in a micro-soxhlet. Following this extraction, which removed the ether-solubles but not the sugars, the tissues samples were freed from ether and extracted with 80% ethanol for 4 hr in the same apparatus. The alcohol extract was concentrated and clarified by the methods of HASSID (1936, 1937), and the total sugars were determined by the use of the anthrone reaction, as previously described (BECK, 1956b). The method developed for estimating RFA concentration in plant tissue will be described in detail in a separate publication. The method involves the chromatographic separation of RFA from interfering impurities contained in the ether extract, and the estimation of RFA concentration by its u.v. absorption at 285 mμ.

RESULTS AND DISCUSSION

Intervarietal resistance

At the time when field surveys of borer activity in south central Wisconsin indicated that the peak of first-generation borer egg hatching had been reached, a series of plant samples was taken from all plots. The tissue samples prepared for bioassay of resistance factors consisted of the portion of the rolled whorl where larval feeding is usually concentrated. This is a section of the whorl which is 4–6 in. in length and is composed of the unexpanded whorl leaves. The interfoliar spaces in this area are quite small, but the leaves are not so tightly packed that the interfoliar water accumulation prohibits borer inhabitation. This portion of the whorl is referred to hereafter as the *borer zone*. At the time of first-generation establishment, parts of the plant other than the borer zone are of little importance, for about 80% of the infesting larvae will be found in this part of the whorl (BEARD and TURNER, 1942; BATCHELDER, 1949; DICKE and PENNY, 1954). At the time of taking this first series of samples, the tassels of all the inbreds were quite small and none had elongated sufficiently to enter the borer zone. The tassel was, therefore, of no direct significance to borer establishment at this growth stage.

The results of the bioassays (*Penicillium*) of this series of samples are presented in Table 1. Highly significant differences were found among the resistance factor activities of the inbreds. The varietal differences in ether-soluble and total activities were in good agreement with field ratings for resistance to leaf feeding. Ether-insoluble resistance factor activity, on the other hand, was low in four of the five varieties tested; only in W210D was there significantly more activity than in the standard susceptible line, WF9. The bioassay results obtained are shown in two different ways in Table 1. The data given in the left-hand columns of the table are the averages of the per cent inhibition observed in the bioassays. Resistance factor activity was detected in the tissue samples of the susceptible lines WF9, and W204, although at a lower level than in the more resistant inbreds. For consideration of activity differences among the inbred lines, resistance activity ratios were calculated and are presented in the right-hand columns of the table. The intervarietal resistance activity ratios facilitate comparison of the resistance factor activity found in a given sample with the comparable sample taken from the standard susceptible inbred, WF9. In the subsequent tables of this section, only the activity ratios are given, rather than the inhibition data from which the ratios were calculated.

As the corn plant grows, the tassel bud elongates to push into and through the borer zone. At the same time, the internodes of the stem tip elongate. This elongation tends to loosen the whorl and widen the interfoliar spaces. Sugar concentrations tend to be higher in the growing tassel than in the surrounding leaves. These changes in the physical and chemical organization of the plant result in an invasion of the tassel structure by many of the borers feeding in the whorl. At this stage of growth, two plant tissues are considered to be the most significant as far as borer feeding is concerned; they are the unexpanded leaves surrounding the tassel, and the tassel they enclose.

TABLE 1—RESISTANCE FACTOR ACTIVITY IN WHORL LEAF TISSUE OF FIVE DENT CORN INBREDS AT THE TIME OF ESTABLISHMENT OF FIRST GENERATION EUROPEAN CORN BORER (NO TASSEL IN BORER ZONE)

Inbred	Average resistance factor activity			Intervarietal resistance activity ratios		
	Ether soluble (%*I*)	Ether insoluble (%*I*)	Total (%*I*)	Ether soluble	Ether insoluble	Total
WF9	20·0	18·2	38·2	1·00	1·00	1·00
W204	22·5	17·7	40·2	1·12	0·97	1·03
W210D	38·2†	41·8†	80·0†	1·91†	2·30†	2·04†
W22	58·2†	24·6	82·8†	2·91†	1·35	2·12†
W22RB	60·7†	16·0	76·7†	3·04†	0·88	2·01†

† Difference from WF9 significant at the 0·01 level of probability.

Plant samples were taken from each experimental plot as the plants therein reached the stage of growth where the tassel extended through the borer zone. Tassel ratios at this sampling were between 50 and 60, and tassel lengths were from 8 to 11 inches. The previous sampling had been timed to coincide with a phenological occurrence external to the plant, namely, the hatching of the borer eggs. The sampling now to be considered was co-ordinated with a growth stage of the plant, namely, the availability of the tassel for borer feeding. The different inbred lines studied all reached this point of growth within about a four-day period. The results of bioassays of borer zone leaves and enclosed tassel are presented in Table 2, where they are expressed as resistance factor activity ratios.

TABLE 2—INTERVARIETAL RESISTANCE FACTOR ACTIVITY RATIOS IN WHORL TISSUES OF DIFFERENT DENT CORN INBREDS IN THE STAGE OF GROWTH AT WHICH BORER LARVAE PENETRATE THE TASSEL

Inbred	Intervarietal resistance activity ratios					
	Rolled leaves			Enclosed tassel		
	Ether soluble	Ether insoluble	Total	Ether soluble	Ether insoluble	Total
WF9	1·00	1·00	1·00	1·00	1·00	1·00
W204	1·38	1·27	1·32	1·29	1·00	1·08
W210D	2·43†	2·38†	2·41†	1·29	1·17*	1·19*
W22	2·50†	1·83†	2·13†	1·12	0·76†	0·86*
W22RB	2·23†	1·84†	2·01†	1·02	0·65†	0·75†

* Difference from WF9 significant at the 0·05 level of probability.
† Difference from WF9 significant at the 0·01 level of probability.

In the rolled leaf samples, varietal differences in ether-soluble resistance factor activity were about the same as in the previous series of samples, except that the activity ratio was higher in W210D and somewhat lower in W22 and W22RB than it had been in the previous series. Marked intervarietal differences in ether-insoluble activity ratios were found; W210D, W22, and W22RB all showed ether-insoluble activity which was greater than that of WF9 to a highly significant degree. In terms of the percentage inhibition of growth observed in the bioassays, the ether-soluble resistance factor activity was lower in this series of leaf tissue samples than it had been in the first sampling. Ether-insoluble activity was about the same in WF9, W204, and W210D as in the previous samples, but showed an increase in W22 and W22RB. The differences in levels of activity found between different samplings are best considered as intravarietal resistance differences and will be discussed in some detail in a later section.

Assays of tassel tissue samples produced very low levels of resistance factor activity in extracts of ether solubles, and the intervarietal resistance activity ratios were not significantly different from WF9 in any of the genetic lines studies (Table 2). The ratios of activity of the ether-insoluble resistance factors showed significant differences, with W210D being just significantly more resistant than WF9, and both W22 and W22RB being more susceptible than WF9 to a highly significant extent. These findings are very important to the problem of host-plant resistance to the borer, for they disclose a serious defect in the supposedly borer-resistant inbreds. In addition to the inbred lines shown in Table 2, unreplicated whorl samples were available of the inbreds Hy, W32, and W182D at the same growth stage. The resistance factor activity found in the tassel tissue of these lines also showed no significant difference from that of WF9. If true of other genetic lines of corn, as well as those used in the present study, the finding of low-resistance factor activity in tassel tissue does much to confirm the conclusions of other workers on the importance of the tassel in the establishment and survival of borer larvae (BEARD, 1941; CAFFREY and WORTHLEY, 1927; BEARD and TURNER, 1942; LUCKMANN, 1956; NEISWANDER and HUBER, 1929; NEISWANDER and POLIVKA, 1930).

Borer larvae tend to remain in the tassel or among the surrounding leaves until the tassel is fully emerged. The opening of the tassel has the effect of leaving many of the borers in exposed positions, and consequently this plant part becomes an unsuitable feeding site. Many of the borers abandon the tassel at this time and move to lower portions of the plant. Further migration from the tassel occurs as this structure matures and dries. The larvae usually find the shelter and feeding conditions they require under leaf sheaths. From this site they feed on sheath, internode, and developing ear structures. As the different inbreds reached the open tassel stage of growth, a series of tissue samples was collected. Samples of internode, leaf sheath, and husks from the developing ears were prepared for bioassay of resistance factor activity. The ear shoots of W210D were too small to allow collection of samples of workable size. Ear shoot samples of W22RB were accidentally destroyed. The bioassays of this series of samples yielded the results shown in Table 3.

TABLE 3—INTERVARIETAL RESISTANCE FACTOR ACTIVITY RATIOS IN LEAF SHEATH, EAR SHOOT, AND INTERNODE TISSUES OF DIFFERENT CORN INBREDS IN THE GROWTH STAGE AT WHICH THESE TISSUES ARE INFESTED BY BORERS VACATING THE TASSEL

Inbred	Resistance factor activity ratios								
	Leaf sheath			Internode			Ear Shoot		
	Ether sol.	Ether insol.	Total	Ether sol.	Ether insol.	Total	Ether sol.	Ether insol.	Total
WF9	1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00
W204	1·06	1·00	1·03	0·90	0·58†	0·72*	0·66†	0·56†	0·61†
W210D	0·73*	0·86	0·79*	0·90	0·95	0·92	—	—	—
W22	1·22	1·03	1·08	2·57†	2·66†	2·62†	1·45†	1·36†	1·40†
W22RB	2·45†	2·79†	2·71†	3·10†	2·63†	2·86†	—	—	—

* Difference from WF9 significant at the 0·05 level of probability.
† Difference from WF9 significant at the 0·01 level of probability.

As indicated by the activity ratios in Table 3, W204 and W210D were no more resistant than WF9 in any category, and were more susceptible in some instances. W22 and W22RB tissues showed some highly significant differences, however. Internode tissue of these two inbreds showed very high activity ratios for both ether-soluble and ether-insoluble resistance factors, there being from 2½ to 3 times the activity shown by internodes of WF9. In respect to leaf sheath resistance, W22 showed no more resistance than that of WF9. W22RB, on the other hand, showed highly significant resistance in the leaf sheaths. These findings are in agreement with those of DICKE and PENNY (1954), who reported that growth and survival of borer larvae in the field was poor on the leaf sheaths of W22R, but that the closely related W22 did not display such sheath resistance. The developing husks of W22 ear shoots showed a low but statistically highly significant resistance, and it is unfortunate that no comparable W22RB samples were available.

TABLE 4—INTERVARIETAL RESISTANCE FACTOR ACTIVITY RATIOS IN THE EAR STRUCTURES OF DIFFERENT FIELD CORN INBREDS AT THE TIME OF ESTABLISHMENT OF SECOND GENERATION EUROPEAN CORN BORER

Inbred	Intervarietal resistance factor activity ratios					
	Husks			Green silks		
	Ether soluble	Ether insoluble	Total	Ether soluble	Ether insoluble	Total
WF9	1·00	1·00	1·00	1·00	1·00	1·00
W204	0·70*	1·09	0·92	0·86	1·51†	1·28*
W210D	1·25*	0·94	1·08	0·91	0·97	0·95
W22	0·99	0·85*	0·91*	0·79	1·38†	1·17*

* Difference from WF9 significant at the 0·05 level of probability.
† Difference from WF9 significant at the 0·01 level of probability.

Table 4 shows the intervarietal resistance factor activity ratios obtained from bioassays of husks and silks from four of the inbred lines. This series of samples was taken at the time when the hatching of second-generation borer eggs was at its seasonal peak in the area. Too few plants remained in the W22RB plots to permit these samples to be collected from this inbred. The results (Table 4) show that the ether-soluble resistance activity was low in the silks of all the varieties. Ether-insoluble activity was higher in silks of W204 and W22 than in WF9 to a highly significant degree. The ether-soluble activity in the husks of W210D was slightly higher than in WF9, but other than this small difference, there was no inbred with appreciable husk resistance. It is quite apparent that neither the husks nor silks of the inbreds tested offered any varietal resistance to borer establishment. In addition to the ear structures, newly hatched borers of the second generation feed on pollen accumulations, midribs, and upper leaf sheaths. Because of the sample size required, it was not practicable to assay pollen and midribs. The leaf sheath activity ratios shown in Table 3 can be taken as an estimation of the resistance of sheath tissue to second-generation establishment. Assays of kernels and cobs have never disclosed any resistance factor activity.

In evaluating the intervarietal resistance found in this study, the question arises as to the agronomic usefulness of some of the smaller differences disclosed, even though they proved statistically significant under the assay conditions employed. Field tests of the resistance of the inbreds to first-generation leaf feeding have resulted in rating WF9 and W204 as highly susceptible, and W210D, W22, W22RB as resistant. The resistance factor activity ratios for total resistance in whorl leaves were not less than 2·00 in the case of any of three resistant lines (Tables 1 and 2). If it is assumed that an activity ratio of 2·00 or more indicates agronomically useful resistance, a rough estimate of general resistance is possible. By such a criterion, it is apparent that the leaves of the borer zone of W210D, W22, and W22RB were resistant to the first-generation establishment, but the tassels of all the inbreds studied were highly susceptible to borer establishment (Table 2). After tasselling, the internodes of W22 and W22RB were borer resistant, but only the leaf sheaths of W22RB displayed any appreciable borer resistance. The ear structures showed no agronomically significant resistance in any of the inbreds tested.

Resistance and growth characteristics

In the introductory section of this paper it was pointed out that corn plants are generally more resistant to borer establishment in the early growth stages (early and mid-whorl) than in later stages (late whorl and tasselling). Such intravarietal resistance is related to the chemical composition of the plant at different stages of growth, and can be correlated with morphological changes in the plant, such as tassel ratio and tassel length. LUCKMANN and DECKER (1952) found that survival of first-generation borers showed a high positive correlation with the tassel ratio of the host plant. They reported little or no borer survival on corn plants having a tassel ratio under 15, and maximum borer survival on plants with tassel ratios of

about 50. LUCKMANN (1956) found that borer survival was also positively correlated with tassel length, and that there was a high correlation between tassel length and tassel ratio. This would, of course, be expected, since tassel length contributes to the tassel height measurement used in the calculation of tassel ratio. In the present study the several plant characteristics routinely measured included both tassel length and tassel ratio. Calculated correlations between the two plant characteristics yielded highly significant coefficients of correlation in plants with tassel ratios of 60 or lower (*r* values were from 0·94 to 0·99 in the different inbred lines).

TABLE 5—GROWTH CHARACTERISTICS OF FIVE DENT CORN INBREDS DURING AND AFTER ESTABLISHMENT PERIOD OF FIRST-GENERATION EUROPEAN CORN BORER

Plant characteristic	Inbred				
	WF9	W204	W210D	W22	W22RB
1. At borer establishment					
Average tassel ratio	10·2	17·4†	18·5†	11·5	11·3
Average tassel length (in.)	0·7	1·9†	2·4†	1·0*	1·2†
Water content of borer zone (per cent)	83·6	81·7	83·1	84·6	85·1
2. Relative maturity rates					
Average tassel growth rate (in./day)	0·80	0·48†	0·55†	0·68	0·63*
Germination to tassel appearance (days)	63	61	60	61	63
Germination to 50 per cent silking (days)	77	71	71	77	78

* Difference from WF9 significant at the 0·05 level of probability.
† Difference from WF9 significant at the 0·01 level of probability.

At the time of first-generation borer establishment, the inbreds used in this study had the characteristics shown in Table 5. If the intravarietal resistance of each of the varieties were identical, that is, no intervarietal differences, it would be expected that the inbred displaying the greatest tassel ratio and tassel length would be the least resistant to borer establishment. On that basis W210D would be the most susceptible of the group and WF9 the most resistant. Both field tests and the data shown in Table 1 have shown W210D to be resistant, and WF9 to be highly susceptible. Tassel ratios and lengths were nearly identical in WF9 and W22, but the two inbreds differ greatly in resistance. NEISWANDER and HUBER (1929) suggested that the growth rate of the tassel was important to resistance, with low growth rate being associated with relative resistance. Tassel growth rates during the period from initial borer establishment to the attainment of a 10 in. tassel length are also shown in Table 5.The highest tassel growth rate was shown by WF9 and the lowest by W204, but both of these inbreds are rated as susceptible lines. The maturity rate of different varieties of corn as determined by the length of the growth period to the silking stage has been claimed to show a positive correlation with relative resistance. The relative maturity rates of the inbreds used are shown in Table 5. It is quite apparent that the relative resistance of the several

inbred lines is not correlated with their relative states of maturity at the time of infestation. Field tests of borer survival and laboratory determinations of resistance factor activity show differences in borer resistance intervarietal in nature and not dependent on the growth characteristics of the inbred lines.

Both intravarietal and intervarietal resistances are clearly demonstrated by the data shown in Table 6. These data show the correlation between tassel length and total resistance factor activity as determined by *Penicillium* bioassay. The two inbreds used were quite similar in tassel length at the time of borer establishment. The assayed tissues in this series were of whorl leaves of the borer zone; no tassel tissues were included in the samples. The resistance factor activity of the leaf tissue declined as tassel length increased in both inbred lines, indicating approximately similar patterns of intravarietal resistance. Throughout the range of tassel lengths the resistance factor activity was approximately twice as high in W22 as in WF9, indicating intervarietal differences in borer resistance.

TABLE 6—CORRELATION OF TOTAL RESISTANCE FACTOR ACTIVITY IN ROLLED WHORL TISSUE OF TWO DENT CORN INBREDS WITH TASSEL LENGTH

Inbred W22		Inbred WF9	
Tassel length (in.)	Total RF activity (%*I*)	Tassel length (in.)	Total RF activity (%*I*)
1·2	113·8	0·7	67·2
2·5	103·8	1·8	43·4
2·9	88·2	7·8	34·2
3·7	74·0	9·0	26·6
10·0	55·0	10·5	28·0
11·2	44·0	11·2	15·6
Correlation coefficient = −0·924*		Correlation coefficient = −0·917*	

* Correlation coefficient highly significant.

It is well established that corn plant resistance to first-generation borer establishment declines as the plant approaches the tasselling stage, and the assay data presented above are fully consistent with this concept. Assays of the resistance factor activity in tissues on which borer larvae feed as the plant matures yielded results indicating that resistance factor concentration is not necessarily at a maximum in the whorl tissues. The data bearing on this point are shown in Table 7, where they are expressed as intravarietal resistance activity ratios. These ratios were calculated in the same manner as the intervarietal ratios discussed earlier, except that the resistance factor activity found in the borer zone at the time of establishment (Table 1) was used as the standard and assigned a value of 1·00. The ratios shown in Table 7 are for the purpose of comparing the total resistance factor activity found in a given tissue of a given inbred with the activity found in the whorl leaves of the *same inbred*.

TABLE 7—TOTAL RESISTANCE FACTOR ACTIVITY IN DIFFERENT TISSUES AND GROWTH STAGES OF DENT CORN INBREDS

Intravarietal resistance activity ratios

Plant tissue and tassel length (TL)	Inbreds				
	WF9	W204	W210D	W22	W22RB
Whorl, borer zone (TL 1–3 in.)	1.00	1·00	1·00	1·00	1·00
Whorl, borer zone (TL 8–11 in.)	0·76†	0·82*	0·89*	0·75†	0·71†
Tassel, borer zone (TL 8–11 in.)	1·39†	1·40†	0·79†	0·55†	0·45†
Internode (TL 14–18 in.)	1·71†	1·18*	0·70†	1·83†	2·20†
Leaf sheath (TL 14–18 in.)	1·51†	1·56†	0·59†	0·77†	2·06†
Husks (TL 14–18 in.)	1·64†	1·02	—	1·06	—
Husks (TL 16–21 in.)	1·71†	1·69†	0·95	0·78*	—
Silks (TL 16–21)	1·95†	2·31†	0·88*	0·98	—

* Difference from 1·00 significant at the 0·05 level of probability.
† Difference from 1·00 significant at the 0·01 level of probability.

The whorl leaves comprising the borer zone in WF9 showed the minimum resistance factor activity, as all other tissues assayed showed highly significant increases in resistance activity. The intravarietal resistance pattern shown by W204 was quite similar to that of WF9. W210D, on the other hand, showed maximum activity in the whorl, and, except for the husks, the other tissues showed significantly less resistance. In W22, young husks and green silks contained the same levels of resistance factor as in the whorl leaves, but the internode was more active to a highly significant degree. Of the W22RB tissues tested, both internode and leaf sheath showed much higher activity than was present in the whorl. The later samples of whorl tissue (tassel length 8–11 in.) were lower in resistance activity than were the earlier samples in all the inbreds. Other than these tendencies, no regular pattern was discerned; the relative activity in the different tissues varied greatly among the several inbreds. The results do not bear out the concept that the plant is necessarily at its maximum resistance during the whorl growth stage. However, it must be pointed out that the assays employed do not allow estimation of what effect the growth stage of borer might have on the manifestation of resistance. It seems quite likely that newly hatched borers would be more susceptible to a given level of resistance factor than older larvae would be.

Identity and distribution of resistance factors

In earlier papers of this series (BECK and STAUFFER, 1957; BECK, 1957; LOOMIS *et al.*, 1957) it was reported that two types of chemical resistance factors had been detected in aqueous extracts of young corn plants. One was soluble in ether and was called Resistance Factor A (RFA). The other type was found to be insoluble in ether and was termed Resistance Factor B (RFB). RFA was isolated (LOOMIS *et al.*, 1957), characterized as 6-methoxy-benzoxazolinone, and synthesized

(SMISSMAN *et al.*, 1956). No other ether-soluble chemicals with resistance activity were detected in the plant concentrates used. At the beginning of the present study it was assumed that all ether-soluble resistance factor activity found in the plant samples was attributable to RFA. The development of a quantitative analytical method for estimating RFA concentration made testing this assumption possible, and it was found that the resistance factor activity of some of the ether extracts was far too high to be attributable to the RFA concentrations detected. It was therefore apparent that there was yet another ether-soluble resistance factor, and it (or they) has been designated Resistance Factor C (RFC). The reason that RFC had not been detected in the earlier work was that in the very young plants used in that work, little or no RFC was present. This point is illustrated by the data of Table 8, discussed below.

TABLE 8—RELATIVE ACTIVITIES OF RESISTANCE FACTORS A AND C IN DIFFERENT CORN PLANT TISSUES

Plant tissue	Variety	Ether-soluble RF activity (%*I*)	Percentage distribution of resistance factor activity	
			RFA %	RFC %
Tassel*	W210D	60·0	100	0
Tassel†	W210D	16·0	0	100
Whorl leaves, borer zone*	W210D	38·2	79	21
Whorl leaves, borer zone†	W210D	25·7	52	48
Internode‡	WF9	29·0	0	100
Internode ‡	W22	70·6	0	100
Husks‡	W22	46·0	39	61
Husks§	W22	28·0	0	100
Silks§	W204	19·0	0	100

* Time of first-generation establishment; tassel below the borer zone.
† Tassel in the borer zone.
‡ Plants at tasselling stage.
§ Time of second-generation establishment.

Bioassays of the ether-soluble resistance factors run on the plant samples employed in this study did not involve a chemical separation of RFA and RFC prior to assay. The assay results were, therefore, a measure of the sum of the activity of the RFA and RFC activities. After the chemical method for estimating RFA became available, the RFA contents of a series of previously bioassayed concentrates were determined. The analytical data, the bioassay results, and a regression of the response of *Penicillium* to pure RFA under standard bioassay conditions provided the information needed for calculation of the relative RFA and RFC activity in the plant concentrates. By this method it was found that RFA was responsible for from 75 to 100% of the resistance factor activity in the ether extracts of whorl samples collected at the time of first-generation borer

establishment. Whorl leaf samples taken at the growth stage where the tassel was within the borer zone showed RFA levels that accounted for from 35 to 65% of the ether-soluble resistance factor activity detected by bioassay. Less than 10% of the ether-soluble activity of tassel samples collected at this growth stage was attributable to RFA. Internode and leaf sheath tissue samples were found to contain only small amounts of RFA at the time the plants tasselled, and the high ether-soluble resistance factor activity of these tissues of W22 and W22RB was apparently due to RFC. Of the inbreds studied, only W22 showed significant RFA activity in the tissues samples at tasselling. The developing husks of W22 at tasselling contained sufficient RFA to register on bioassay. At this sampling, however, W22 ear shoots were more immature than those of the other inbreds. These results are shown in Table 8. Although chemically detectable amounts of RFA were found in all tissues analysed, RFA occurred in amounts which were significant to borer resistance only in the comparatively early growth stages of the plant parts. The tissue concentration of Resistance Factor C, on the other hand, appeared to be less dependent on the developmental stage of the plant, and to account for most or all of the ether-soluble resistance factor activity of the plants beyond the late-whorl growth stage.

Nothing is known, at the present time, of the chemical properties of either RFB or RFC. There is some indication that the active principle in RFB consists of two different compounds, but this is not yet established. There is also some evidence that the active compounds in RFC and RFB concentrates are chemically or functionally related. In those plant samples where RFA was not responsible for the largest part of the resistance factor activity, the activity was equally divided between RFC and RFB. The exception to this generalization was found in tassel tissue, where ether-insoluble activity (RFB) was higher than the ether soluble.

Resistance Factor A appears to be of primary importance to the initial attack of the first-generation larvae of the European corn borer. RFB may contribute significantly to resistance to leaf feeding in some cases (as in W210D, see Table 1), but RFC is not present in significant amounts (Table 8). The importance of RFA to the resistance of later growth stages of the corn plant is probably negligible, as its concentration in tissues which are significant from the standpoint of borer infestation declines rapidly as the tissues mature. The progressive accumulation of sugars in the tissues in which the borers feed (Beck, 1956b), and the inverse relationship between RFA activity against the borer and tissue sugar concentration (Beck, 1957) also tend to lessen the possible importance of RFA as a factor in border resistance beyond the leaf-feeding stage.

Specificity of resistance

The finding that the chemical resistance factors in corn plant tissue have a growth-inhibiting effect on a variety of organisms (Beck and Stauffer, 1957) brings up the question of the specificity of resistance. Correlations between resistance of corn to different organisms have been observed in some cases. Huber and Stringfield (1940, 1942) found a significant positive correlation

between varietal resistance to borer leaf-feeding and resistance to the corn-leaf aphid, *Aphis maidis* Fitch. The aphids attack the whorl leaves and the emerging tassel. WF9 has been found to be quite susceptible and W22 resistant to corn-leaf aphid (HUBER and STRINGFIELD, 1940; SNELLING *et al.*, 1940; COON, 1948 COON *et al.*, 1948). This is in agreement with the inbreds' rated resistance to borer leaf feeding and is also in agreement with the resistance factor activity assay results given in Table 1. In the early plant growth stages, the corn-leaf aphid and the European corn borer attack essentially the same plant parts, and resistance to the two forms appears to be similar. Resistance to the corn earworm, *Heliothis armigera* (Hübn.), involves the physical and chemical characteristics of the ear structure, and it would hardly be expected that resistance to earworm establishment would be correlated with whorl resistance to borer leaf feeding. Although chemical and nutritional factors have been postulated as of possible importance in earworm resistance (COLLINS and KEMPTON, 1917; POOLE, 1936; DOUGLAS, 1947), physical factors associated with husk and kernel characteristics may also be involved (DITMAN and CORY, 1933; DICKE and JENKINS, 1945; and others). Whereas WF9 is highly susceptible to European corn borer, it is considered somewhat resistant to corn earworm (BLANCHARD *et al.*, 1941). Resistance factor activity in the husks and silks was found to be much higher than in the whorl tissue of WF9 (Table 7). This difference in tissue concentration of resistance factors may account for the difference in resistance towards the two insects. There may also be a similarity between resistance to insects and resistance to plant pathogens. JOHANN and DICKSON (1945) detected the presence of a *Diplodia zeae* growth inhibitor in corn-plant tissue. The inhibiting substance they described apparently is the same as Resistance Factors A or C. Resistance Factor A was isolated as an antifungal agent in corn by VIRTANEN *et al.* (1956), and a closely related compound as a *Fusarium* resistance factor in rye (VIRTANEN and HIETALA, 1955). The Wisconsin inbreds W22 and W22RB were developed as stalk-rot resistant lines, and were subsequently found to be borer resistant. In general, it seems probable that the resistance of corn to different attacking organisms has a common causal basis and its manifestation depends on (1) the tissue concentrations of resistance factors, (2) the correlation of resistance factors distribution with the establishment sites of the parasitic organism, and (3) the sensitivity of the infesting organisms (insect or pathogen) to any or all of the several resistance factors.

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THE DIFFUSION OF NON-ELECTROLYTES THROUGH THE ISOLATED CUTICLE OF *SCHISTOCERCA GREGARIA*

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Abstract—The diffusion of seven non-electrolytes through isolated abdominal cuticle of *Schistocerca gregaria* has been investigated. The permeability of the epicuticular wax layer was calculated and shown to be related to the solubility of the penetrating molecules. The permeability of the whole cuticle was found to deviate from this relation with the more rapidly penetrating compounds. The deviation of the whole cuticle from the linear relation with solubility was caused by the appreciable resistance to diffusion offered by the cuticle beneath the wax layer. This resistance was found to be proportionally greater for the more rapidly penetrating compounds, up to half being contributed by the lower layers of the cuticle. The diffusion of compounds through the cuticle beneath the epicuticular wax layer was essentially similar to that through liquid filled spaces larger than the diameter of the diffusing molecules. This diffusion was only some 0·24% of that through an equivalent unstirred water layer, indicating that the total area available for free diffusion is small.

INTRODUCTION

THE permeability of insect cuticle has been the subject of several extensive studies during the recent decade. The phenomenon of transpiration and, in particular, the rôle of the wax layers in waterproofing the insect cuticle has been elucidated in a series of investigations (WIGGLESWORTH, 1945; BEAMENT, 1945; HOLDGATE and SEAL, 1956). The mechanism of penetration of other compounds is, as RICHARDS (1951) has pointed out, less well understood and on the basis of the published work much of our knowledge is still conjectural. Much of what is known about the processes involved in the passage of molecules through membranes is based on comparative studies using selected series of compounds and it seems clear that our knowledge of cuticle permeability would benefit from such a study. Previous investigations on the penetration of substances through cuticle have usually relied on various biological and toxicological techniques (ALEXANDROV, 1935; HURST, 1943; WEBB and GREEN, 1945) and, though much useful information has emerged, can only be regarded as qualitative in nature. The quantitative study of RICHARDS and FAN (1949) on the permeability of the isolated cuticle of *Phormia* is a notable exception, but these authors were largely concerned with the variation in permeability to the substances studied. The present investigation on the diffusion of a series of non-electrolytes through isolated cuticle of *Schistocerca gregaria* was therefore undertaken in an attempt to determine some of the physico-chemical factors involved in the penetration of cuticle.

METHODS

The diffusion of material through the cuticle was studied by exposing isolated cuticle to an aqueous solution of the test substance and determining by chemical analysis the amount which had penetrated the cuticle.

The cuticle used in this work was that of the third abdominal tergum of adult female *Schistocerca gregaria* Forskål. Cuticle from this region was carefully removed and freed from underlying organs. Care was taken to avoid abrasion of the epicuticle or damage to the epidermis which was left intact in this preparation. The epidermal surface was then washed in a gentle stream of saline before mounting in the diffusion apparatus.

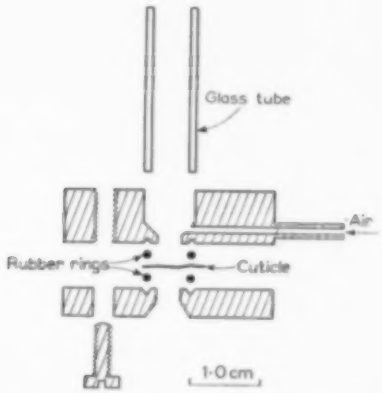

FIG. 1. A diagrammatic vertical section of the apparatus used to measure the diffusion through isolated cuticle. The hatched areas represent the portions which were made of brass.

In the diffusion apparatus, which is illustrated in Fig. 1, the cuticle was held in position by two rubber " Gaco " rings seated in V-shaped grooves (BEAMENT, unpublished). When the apparatus was assembled the cuticle formed the floor of a cell which contained oxygenated and stirred saline. During the experiment that apparatus stood in a large volume (*c.* 1 litre) of the test solution so that the lower (epicuticular) surface of the cuticle was in contact with this solution. The area of cuticle exposed to the test solution was 0·283 cm^2 and the volume of the saline contained in the cell 1·0 ml. The test solutions were made up with saline, so that the cuticle was bathed on both sides by oxygenated and stirred saline and only differed in the addition of the test substance to the outer solution. The composition of the saline used was that given by HOYLE (1955). The experimental temperature was $20{\cdot}0 \pm 0{\cdot}5°$ C.

At the end of each experiment the contents of the cell was removed and analysed to determine the concentration of the test substance. Ethanol was determined by the method of CONWAY (1950), thiourea and derivatives by the method of CHESLEY (1943), and urea and derivatives by the method of BARKER (1944).

RESULTS

The amount of material diffusing through the cuticle was measured by determining the concentration of the test substance at the inner surface of the cuticle. A permeability constant could then be calculated according to Fick's Law from the equation :

$$P = \frac{V}{At} \ln \frac{C_o - C_i}{C_o},$$

where C_i and C_o are the concentrations of the test substance above and below the cuticle at time t after application. A is the area of cuticle exposed and V the volume of saline inside the diffusion cell. P has the dimensions cm sec^{-1}.

TABLE 1

External concentration of ethanol	Time	$P \times 10^6$ cm sec^{-1}	Mean ± S.E.
0·1 M	20 hr	1·171 0·996 1·610 0·891 0·914	1·116 ± 0·141
1·0 M	1 hr	0·928 1·357 1·654 1·527 0·689	1·231 ± 0·282
1·0 M	20 hr	0·834 0·966 1·185 1·562 1·477 1·725 1·427 1·106	1·285 ± 0·291

The permeability constants obtained for the diffusion of ethanol from external concentrations of 0·1 and 1·0 M are given in Table 1. It will be seen that the values remain substantially constant despite differences in time and external concentration of ethanol. Thus the constant P appears to be a valid measure of the resistance to free diffusion offered by the cuticle.

The permeability constants obtained for the seven non-electrolytes investigated are summarized in Table 2. The values for the diffusion through whole cuticle have been compared with those in which the epicuticular wax was removed by immersing the insect for 2 minutes in boiling chloroform before removing and mounting the cuticle. Cuticles treated in this way showed a general blackening on immersion in 5% ammoniacal silver nitrate. The values for the whole cuticle show a considerable range of permeability from $1{\cdot}223 \times 10^{-6}$ cm sec^{-1} for ethanol

TABLE 2

Compound	Whole cuticle		Chloroform treated cuticle
	C_o	$P \pm$ S.E. (n) $\times 10^7$ cm sec^{-1}	$P \pm$ S.E. (n) $\times 10^6$ cm sec^{-1}
Ethanol	0·1—1·0 M	12·23 ± 0·23 (18)	2·47 ± 0·43 (6)
Phenyl thiourea	0·015 M	4·72 ± 0·65 (5)	1·06 ± 0·14 (8)
m-Tolyl urea	0·020 M	4·10 ± 0·35 (5)	1·10 ± 0·13 (5)
Diethyl thiourea	0·10 M	1·80 ± 0·34 (5)	1·43 ± 0·08 (6)
Thiourea	0·20 M	0·66 ± 0·08 (14)	1·91 ± 0·19 (10)
Dimethyl urea	0·50 M	0·29 ± 0·03 (6)	1·14 ± 0·02 (7)
Urea	0·50 M	0·19 ± 0·004 (7)	1·68 ± 0·28 (5)

to $1{\cdot}890 \times 10^{-8}$ cm sec^{-1} for urea. The chloroform-treated cuticles yielded higher values, but these ranged only between $1{\cdot}06 - 2{\cdot}47 \times 10^{-6}$ cm sec^{-1}. The cuticular wax removed by the chloroform treatment does, therefore, offer an appreciable resistance to diffusion for all the compounds studied. The resistance to diffusion of the cuticle remaining after chloroform treatment, expressed as a percentage of that through the whole cuticle, is: ethanol, 49·5%; phenyl thiourea, 44·4%; *m*-tolyl urea, 37·2%; diethyl thiourea, 12·6%; thiourea, 3·4%; dimethyl urea, 2·6%; urea, 1·1%. Thus the more rapid the penetration the greater the resistance to diffusion offered by the cuticle components remaining after chloroform treatment. With a slowly penetrating molecule such as urea diffusion is effectively controlled by the cuticular wax; with a rapidly penetrating molecule like ethanol approximately half the resistance to diffusion is contributed by the remainder of the cuticle.

A permeability constant for the wax layer removed by the chloroform treatment can be calculated from the equation:

$$P_1 = \frac{P P_2}{P_2 - P},$$

where P is the permeability of the whole cuticle and P_2 that after dewaxing with chloroform. This assumes that the chloroform merely removes the cuticular wax and does not significantly affect the remainder of the cuticle. The validity of this

TABLE 3

Compound	Calculated permeability of wax layer (cm sec^{-1})
Ethanol	$2{\cdot}42 \times 10^{-6}$
Phenyl thiourea	$8{\cdot}49 \times 10^{-7}$
m-Tolyl urea	$6{\cdot}52 \times 10^{-7}$
Diethyl thiourea	$2{\cdot}06 \times 10^{-7}$
Thiourea	$6{\cdot}81 \times 10^{-8}$
Dimethyl urea	$3{\cdot}00 \times 10^{-8}$
Urea	$1{\cdot}91 \times 10^{-8}$

assumption has been tested and will be dealt with subsequently. The calculated permeability constants for this wax layer are given in Table 3.

In Fig. 2 the permeability constants for the whole cuticle and the calculated

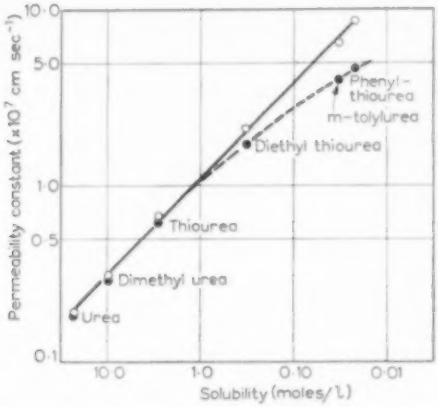

FIG. 2. The calculated permeability of the epicuticular wax layer (open circles) and that of the whole cuticle (closed circles) related to the solubility of the penetrating compounds (in aqueous solution at 20° C).

values for the wax component have been plotted against the solubility of the penetrating compounds in water at 20° C. The values for the wax component tend to fall along a straight line while those for the whole cuticle tend to deviate with increasing solubility.

In order to test more fully the effects of chloroform treatment the diffusion through abraded cuticles has been compared with that through chloroform-treated cuticles. To do this adult female *Schistocerca gregaria* were anaesthetized with CO_2 and, after removal of the wings, were placed for varying periods in a

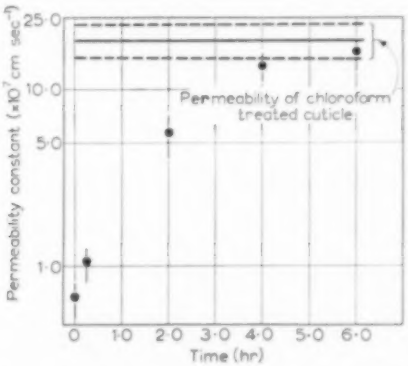

FIG. 3. The effect of abrasion with "Almicide" dust on the permeability of the cuticle to thiourea. The vertical lines indicate the extent of twice the standard error, while the horizontal broken lines indicate that for the chloroform treated cuticle.

mechanically rotated glass jar containing "Almicide" dust. The time of this treatment was used as a measure of the degree of abrasion of the cuticle. After abrasion the cuticle was removed, mounted in the apparatus, and the diffusion of thiourea from an external solution of 0·05 M was studied. The results obtained are illustrated in Fig. 3, and it will be seen that the abrasion caused a marked increase in permeability, rising after 4 hours to a value close to that of chloroform-treated cuticles. Control experiments showed that abrasion of the chloroform-treated cuticle had no significant effect on the permeability:

Permeability of chloroform-treated cuticle: $1{\cdot}91 \pm 0{\cdot}19\ (9) \times 10^{-6}$ cm sec^{-1}
Permeability of abraided and chloroform-treated cuticle:
$1{\cdot}93 \pm 0{\cdot}21\ (8) \times 10^{-6}$ cm sec^{-1}

It therefore seems reasonable to assume that after 4 hours the "Almicide" had effectively disrupted the wax layer, which was also removed by chloroform, and that the effects of the chloroform on the remainder of the cuticle were small as far as its permeability was concerned.

In order to study the nature of the diffusion process through chloroform-treated cuticles the permeability of these have been compared with the molecular weight of the penetrating compounds (Table 4). The product of the permeability constant and the square root of the molecular weight, $PM^{\frac{1}{2}}$, is also included, for comparison of $PM^{\frac{1}{2}}$ for several compounds throws light on the type of diffusion process involved in the passage of a substance through a membrane (Davson and Danielli, 1952). It will be seen that although there is a tendency for the smaller molecules to diffuse most rapidly, the values of $PM^{\frac{1}{2}}$ remain relatively constant and do not appear to be related to the size of the diffusing molecule.

Table 4

Compound	Molecular weight	$P \times 10^6$ cm sec^{-1}	$PM^{\frac{1}{2}} \times 10^5$ cm sec^{-1}
Ethanol	46·07	2·47	1·67
Urea	60·06	1·68	1·30
Thiourea	76·12	1·91	1·66
Dimethyl urea	88·11	1·14	1·07
Diethyl thiourea	132·22	1·43	1·64
m-Tolyl urea	151·19	1·10	1 35
Phenyl thiourea	152·21	1·06	1·31

DISCUSSION

It has been shown, by comparison with dust-abraided cuticles, that removal of the epicuticular wax with hot chloroform has little effect on the permeability of the remainder of the cuticle. From this it was concluded that the calculated permeability of the wax layer, which depends in part on that of the remainder of the cuticle, was a reasonable approximation. The permeability constants for this wax layer have been shown to parallel the solubility in water of the penetrating molecules. As the solubility of a substance can be related to its chemical potential

and also to its partition coefficient (FERGUSON, 1939) it is reasonable to conclude that diffusion through the wax layer is largely determined by phase distribution relationships.

Diffusion through the whole cuticle, however, does not show a linear relationship with solubility but deviates markedly with the more rapidly penetrating compounds. This deviation is due to the resistance to diffusion of the cuticle beneath the epicuticular wax layer which has been shown to be proportionally greater for the more rapidly diffusing molecules. With a small lipoid soluble molecule such as ethanol only half the resistance to diffusion is due to the wax layer. Thus diffusion of molecules through this cuticle is not, as is sometimes supposed, necessarily controlled exclusively by the epicuticular wax. It is of interest in this respect to consider the theory of FERGUSON (1939, 1951) which relates the toxicity of a compound to its thermodynamic activity and also, as an approximation, to its solubility. In this case the effect of the increased resistance to diffusion of the lower layers of the cuticle may well cause a deviation from this theory when the diffusion of the substance through the cuticle is a rate-limiting process in the mechanism of toxicity. WIGGLESWORTH (1942) has recorded a correlation between the thickness of the endocuticle and the speed of action of pyrethrins in *Rhodnius*. The pyrethrins are lipoid soluble substances which might be expected to penetrate relatively rapidly and it is possible that diffusion is similar in this respect to that through the cuticle of *Schistocerca*.

The comparison of the rates of diffusion through chloroform-treated cuticles showed that although the smaller molecules tended to diffuse more rapidly the product of the square root of the molecular weight and the permeability constant, $PM^{\frac{1}{2}}$, remained relatively constant. It is known that for the diffusion through water of molecules up to the size of hexoses the product $DM^{\frac{1}{2}}$ is approximately constant, while DAVSON and DANIELLI (1952) have shown that with a large pored membrane the product $PM^{\frac{1}{2}}$ also remains constant, but falls off with increasing molecular size with a small pored membrane. Thus diffusion through the layers beneath the epicuticular wax is similar to that through liquid-filled spaces which are significantly larger than any of the diffusing molecules. DAVSON and DANIELLI (1952) quote values of $DM^{\frac{1}{2}}$ for the diffusion of non-electrolyte molecules through water which are approximately constant at $7{\cdot}05 \times 10^{-5}$ cm^2 sec^{-1}. Taking the thickness of the cuticle and epidermis to be approximately 120μ and the mean value of $PM^{\frac{1}{2}}$ as $1{\cdot}428 \times 10^{-5}$ cm sec^{-1}, then it is possible to calculate a comparable value of $1{\cdot}714 \times 10^{-7}$ cm^2 sec^{-1} for the cuticle. Thus diffusion through the layers of the cuticle beneath the wax layer is only approximately 0·24% of that through an equivalent unstirred water layer. It appears therefore that although diffusion is similar to that through relatively large liquid-filled spaces the area available for free diffusion is small.

Diffusion through the lower layers of the cuticle involves penetration of a cellular layer, the epidermis. It is, therefore, somewhat surprising that $PM^{\frac{1}{2}}$ should yield approximately constant values, for it might be expected that passage through a cellular layer would involve diffusion through some sort of lipoid layer

or layers. There are several possible explanations. It is possible that the relatively impermeable layers overlying the epidermis mask any selective effects exerted by the epidermal cells. Alternatively it may be that penetration of the epidermis involves diffusion through intercellular spaces or through " pores " in the cell-walls. The possibility also exists that, despite the care taken to avoid damage, any selective effect exerted by the epidermis might be upset by the experimental conditions.

In their investigation on the permeability of the isolated cuticle of the larva of the blowfly, *Phormia regina*, RICHARDS and FAN (1949) were largely concerned with the variation in the values obtained. They recorded wide variations from individuals reared under the same conditions, from up to X8 for urea to X18 for cobalt chloride. These variations were paralleled by similar variations in electrical impedance and toxicological response and, although these do not constitute conclusive proof, it seems likely that this variation was valid and not the result of experimental errors. The variation in permeability of the cuticle of *Schistocerca* rarely exceeded a factor of two and was never more than a factor of three (cf. Table 1). RICHARDS and FAN attribute their variation to hypothetical variations in one or more of the component layers of the cuticle. It seems possible, however, that the permeability could equally well be affected by contamination of the cuticle by the organic media in which the larvae were reared, for it has been shown by HOLDGATE (1955) that such insects show wide variations in angle of contact which persist even after washing with a variety of solvents. The smaller variation obtained for the cuticle of *Schistocerca* could be due to this insect's mode of life, for it would be less liable to contamination than one which burrows in carrion.

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VERGLEICHEND HISTOLOGISCHE UNTERSUCHUNGEN AN NORMALSENSIBLEN UND GEGEN DDT-SUBSTANZ RESISTENTEN STÄMMEN VON *MUSCA DOMESTICA* L.*

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Abstract—Between strains of *Musca* susceptible and resistant to DDT-active ingredient, differences have been found in the lipoid and lipoprotein content of the epicuticle and the total cuticle and also the epidermal cells of the tarsi and the thorax. Another divergence has been observed in the amount secreted by the tarsal gland in the fifth segment. On both tarsal nerves of each segment there are structures that appear to be two motor ganglia cells.

EINLEITUNG UND PROBLEMSTELLUNG

Musca domestica hat zusammen mit anderen hygienisch bedeutungsvollen Insekten durch einen Selektionsprozeß an den verschiedensten Orten der Erde gegenüber den auf der Basis der chlorierten Kohlenwasserstoffe aufgebauten synthetischen Insektiziden Resistenz entwickelt (METCALF, 1955; WIESMANN, 1955a). Das Wesen der Insektizidresistenz, die zu einem schwerwiegenden praktischen Problem wurde, zu erkennen, ist in vielen Laboratorien der alten und neuen Welt Gegenstand eingehender Forschungen. Auch in den wissenschaftlichen Laboratorien der J. R. GEIGY A.G., BASEL, sind wir in einer kleinen Arbeitsgruppe bestrebt, die mannigfaltigen Mechanismen, durch die die Insektizidresistenz bedingt wird, kennen zu lernen. Unsere eigenen Untersuchungen beschränken sich vorläufig hauptsächlich auf das Resistenzproblem bei *Musca domestica* gegenüber dem Dichlordiphenyltrichloräthan, der DDT-Substanz.

Nach der Literatur (STERNBURG und KEARNS, 1950; 1953; TAHORI und HOSKINS, 1953) und nach eigenen Untersuchungen (BRUTTIN und WIESMANN, in Vorbereitung; REIFF, 1955a) ist bekannt, daß die gegen die DDT-Substanz resistenten Fliegenstämme die durch die Cuticula resorbierte Substanz meist in höherem Maße enzymatisch abbauen und dadurch zu entgiften imstande sind, als die sensiblen Fliegen (REIFF, 1955b). Meist verbleibt aber im Körper der resistenten Fliegen, im folgenden *R*-Fliegen genannt, eine nicht unbedeutende Menge nicht abgebauter DDT-Substanz, die die Tiere, ohne Vergiftungssymptome zu zeigen, tolerieren können (BRUTTIN und WIESMANN, in Vorbereitung; WIESMANN und REIFF, 1956). Uns interessierte zu wissen, durch welche Mechanismen die *R*-Fliegen diese Toleranz zustande bringen. Als solche erkannten wir bis anhin die stark erhöhte Schwelle der Empfindlichkeit des Nervengewebes gegenüber der DDT-Substanz, die geringere Permeabilität desselben (WIESMANN, 1955b), den erhöhten und anders zusammengesetzten Lipoidgehalt der Cuticula und des Gesamtkörpers, sowie auch des Nervensystems, die zu einer starken Blockierung der lipophilen DDT-Substanz fähig sind (WIESMANN und REIFF, 1956). Zudem

* Grundlagen zur Resistenzforschung, 8. Mitteilung.

ergab sich, daß unter dem Einfluß der DDT-Substanz ein Metabolismus der Lipoide eintritt, der es den *R*-Fliegen ermöglicht, die Blockierung der DDT-Substanz noch zu erhöhen, während dieser Metabolismus bei den *S*-Fliegen gerade gegenteilige Folgen hat (WIESMANN und REIFF, 1956).

Auf Grund dieser fundamentalen Erkenntnisse interessierte uns zu wissen, ob auch histologische Unterschiede zwischen den *R*- und *S*-Fliegen in den verschiedenen Organsystemen festzustellen wären, die mit der Aufnahme der DDT-Substanz in Beziehung stehen resp. als Erfolgsorgane der Substanz eine Rolle spielen. Als solche kommen in Frage die Tarsen, die Körperhaut, ferner auch das Nervensystem. Es erscheint nicht ausgeschlossen, daß durch diese histologischen Untersuchungen weitere Beiträge zum Verständnis der Insektizidresistenz erhalten werden könnten.

Als Untersuchungsmaterial dienten uns zwei normalsensible (*SN* und *SK*) und zwei hochresistente Stämme (*R* und *RK*) der Stubenfliege.

MORPHOLOGIE UND HISTOLOGIE DER TARSEN

Die Stubenfliege berührt, wie Laufspuren auf berußtem Glanzpapier ergeben haben, beim Gehen die Unterlage nur mit den Pulvillen und den letzten drei Tarsengliedern. Beim Stillestehen ruht die Fliege meist auf allen 5 Tarsengliedern, eventuell sogar noch auf der Tibia. Eine weitere Berührung des Körpers mit der Unterlage erfolgt nicht. Die Tarsen sind demnach auch die Orte, mit denen die Fliege auf einer mit einem Dauerkontaktinsektizid behandelten Oberfläche in der ersten Zeit in Berührung kommt, und sie stellen demzufolge auch die Haupteintrittspforten für diese Stoffe dar. In ihnen erfolgen auch die ersten Vergiftungsprozesse, und wie wir feststellten (WIESMANN und REIFF, 1956) spielen sich hier auch die ersten Regulationsreaktionen bei Aufnahme des Insektizides durch den Kontakt ab.

Die Tarsen von *Musca* bestehen aus 5 Segmenten oder Tarsomeren (s. Abb. 1) von ungleicher Größe. Das erste Segment ist das längste, das 2. und 5. sind ca. gleich groß und das 3. und 4. sind die kürzesten, wenig länger als breit. Der Prätarsus am Ende des 5. Tarsensegments besteht aus einem Paar Klauen und langen, basolateralen Pulvillen. Zwischen den Tarsen der Männchen und Weibchen bestehen keine Unterschiede, und auch zwischen den *S*- und *R*-Fliegenstämmen, die miteinander verglichen wurden, konnten, rein morphologisch, keine gesicherten Unterschiede festgestellt werden. Dagegen unterscheiden sich die Tarsen der *S*- und *R*-Fliegen, wie im folgenden gezeigt werden soll, in physiologisch-biochemischer und z.T. auch in histologischer Beziehung deutlich voneinander.

Die Cuticula

Strukturelle Unterschiede zwischen der Tarsen-Cuticula der *R*- und *S*- Fliegen liessen sich lichtoptisch nicht feststellen. Dagegen konnte man nach Behandlung der Tarsen mit verschiedenen Agenzien recht interessante

Unterschiede auffinden, die wahrscheinlich in einem engen Zusammenhange mit der Resistenz stehen.

Kurze Behandlung der Tarsen mit Lipoidlösungsmitteln. Taucht man unbehandelte Extremitäten der *S*- und *R*-Fliegen in Wasser, dann findet an keinem der Beinglieder eine Benetzung statt. Das Wasser läuft von den Extremitäten sofort ab.

Werden nun, durch Blausäure abgetötete, *S*- und *R*-Fliegen für 1, 5, 10 oder 15″ in Azeton und hierauf nach kurzem Trocknen, mit den Extremitäten in destilliertes Wasser eingetaucht, dann kann man, wie aus Tabelle 1 ersichtlich ist, folgende interessante Unterschiede feststellen.

TABELLE 1

Eingetaucht in Azeton	Wasserbenetzung der Tarsen von je 20 Fliegen (10♀, 10♂)					
	S-Fliegen (Stamm *SN* + *SK*)		*R*-Fliegen			
	Zahl der Fliegen	%	Zahl der Fliegen		%	
			R	*RK*		
1″	20	100	0	0	0	
5″	20	100	0	0	0	
10″	—	—	5	7	25	35
15″	—	—	20	20	100	100

Bei den *S*-Fliegen der beiden Stämme genügt schon ein ganz kurzes Eintauchen der Extremitäten in Azeton, um nachher eine Benetzung derselben durch Wasser zu erhalten. Die *R*-Fliegen dagegen benötigen hiezu eine Tauchzeit von mindestens 10″, um einzelne Tarsen benetzbar zu machen und 15″, um bei allen Individuen diesen Effekt zu erzielen. Die beiden *R*-Stämme verhalten sich praktisch gleich. Gleiche Ergebnisse erzielt man auch bei der Kurzbehandlung der Tarsen mit anderen Lipoidlösern, wie Aethyläther, Petroläther, Essigäther etc.

Das unterschiedliche Verhalten der Tarsen der *R*- und *S*-Fliegen in ihrer Benetzbarkeit nach einer Kurzbehandlung mit Lipoidlösungsmitteln deutet nicht nur auf eine unterschiedliche Löslichkeit der Lipoide der Epicuticula hin (WIESMANN und REIFF, 1956), sondern, wie die Untersuchungen von REIFF (1955b) ergaben, handelt es sich auch um einen quantitativen Unterschied dieser Lipoide. Dieselben sind bei den *R*-Fliegenstämmen 30–40% größer als bei unseren *S*-Stämmen. Die kleinen Lipoidmengen der Epicuticula der Tarsen der *S*-Fliegen sind schon nach einer Sekunde Tauchen in Azeton abgelöst, während die stärker entwickelte Lipoidschicht bei den *R*-Fliegen deutlich länger braucht, bis sie weggelöst ist und dadurch eine Benetzung der Tarsen möglich wird.

Behandlung der Tarsen mit Diaphanol. Bei weiteren Versuchen wurde festgestellt, daß die Tarsen und übrigen Extremitätenglieder bei den *S*- und *R*-Fliegenstämmen unterschiedliche Mengen von Inkrusten aufweisen.

Die Extremitäten der *S*- und *R*-Fliegen wurden während einer Woche bis zur völligen Entpigmentierung in Diaphanol eingelegt. Das Diaphanol zerstört nicht

nur die Pigmente, sondern es löst aus der Cuticula auch die Inkrusten, meist Proteine und Lipoproteine, heraus und macht die vorher harte, schlecht schneidbare Cuticula geschmeidig (FRAENKEL und RUDALL, 1940). Die so behandelte Cuticula besteht nur noch aus reinem Chitin, das die entsprechenden Reaktionen zeigt.

Die mit Diaphanol behandelten Beine wurden nach eintägigem Wässern in fliessendem Wasser in eine 1%ige, wässrige Neutralrotlösung gelegt und während 48 Stunden durchgefärbt, sodaß auch die Hohlräume in den Tarsen mit der Farbe ausgefüllt waren. Neutralrot ist bekantlich ein Indikator, der bei einem pH von 6,8 eine rote Farbe aufweist, die bei pH 8,0 in Gelb umschlägt.

Die mit Neutralrot durchgefärbten Extremitäten wurden nach kurzem Wässern in eine 1/10 N Na(OH)-Lösung eingelegt. Die Na(OH)-Lösung diffundiert ziemlich leicht durch die mit Diaphanol entkrustete Cuticula und erzeugt dann an der Neutralrotlösung in den Tarsenräumen einen sehr gut sichtbaren Farbumschlag in Gelb. Die Zeit bis zum Eintreten des Farbumschlages wurde jeweils gemessen. Die Resultate sind aus Tabelle 2 zu entnehmen.

TABELLE 2—ZEIT BIS ZUM EINTRITT DES FARBUMSCHLAGES VON ROT IN GELB BEI JE 60 EXTREMITÄTEN. DURCHSCHNITTSWERTE

S-Fliegenstämme		*R*-Fliegenstämme	
SN	80 Sekunden	*R*	34 Sekunden
SK	83 Sekunden	*RK*	42 Sekunden

Nach diesen Zahlen ist die Durchlässigkeit der entkrusteten Cuticula für die 1/10 N Na(OH)-Lösung bei den *R*-Fliegenstämmen rund 2 mal größer als bei den *S*-Fliegen. Dies bedeutet wohl, daß die Tarsen der *R*-Fliegen deutlich mehr Inkrusten, also Proteine und Lipoproteine aufweisen als die *S*-Fliegen.

Der erhöhte Protein- und Lipoproteingehalt der Cuticula bei den *R*-Fliegen steht wahrscheinlich im Zusammenhang mit der stärkeren Blockierungsfähigkeit der *R*-Tarsen gegenüber der lipophilen DDT-Substanz, worauf bereits die von REIFF (1955b; 1956) auf Grund anderer Methoden erhaltenen Resultate hingewiesen haben (s. auch WIESMANN und REIFF, 1956). In gleicher Richtung hat sicherlich die quantitativ und qualitativ unterschiedliche Zusammensetzung der epicuticularen Lipoide ebenfalls eine Bedeutung (WIESMANN und REIFF, 1956). Sie helfen ebenfalls mit, die in die Tarsen eindringende DDT-Substanz zu blockieren.

Die Hypodermis der Tarsen

Die Hypodermis, der lebende Teil des Tarsenintegumentes, besteht aus einem einschichtigen Zellepithel. In der histologischen Beschaffenheit dieser Zellen sind zwischen den *S*- und *R*-Fliegenstämmen Unterschiede gefunden worden, die sehr wahrscheinlich mit den oben besprochenen Unterschieden im Lipoidgehalt der Epicuticula und auch der Gesamttarsen im Zusammenhang stehen.

Die Extremitäten von je 5 männlichen und 5 weiblichen Fliegen der 4 Fliegenstämme wurden nach Amputation in Carnoy fixiert, in Paraffin eingeschlossen und geschnitten. Die 7 μ dicken Schnitte wurden mit Böhms Haematoxylin-Orange G gefärbt und in Canadabalsam übergeführt. Es wurden hauptsächlich Längsschnitte hergestellt.

Tarsale Hypodermiszellen der S-Fliegen (Abb. 2). Bei den *S*-Fliegen besteht das Tarsenepithel aus gleichmäßig, flach prismaförmigen Zellen mit runden Zellkernen, die mit wenig, aber grossen Chromatinschollen ausgestattet sind. Das Zellplasma erscheint als feinkörnige, kompakte Masse. Die etwas größeren Haarbasiszellen weisen gleiche Strukturen auf, und sie sind in die Hypodermis lückenlos eingefügt.

Tarsale Hypodermiszellen der R-Fliegen (Abb. 3). Die *R*-Fliegen besitzen etwas größere Hypodermiszellkerne, mit vielen runden Chromatinbrocken. Das Zellplasma ist nicht kompakt wie bei den *S*-Fliegen, sondern es weist größere und kleinere Vacuolen auf, die der Cuticula genähert, oder sogar angelagert sind. Besonders stark und auffallend ist diese Vacuolenbildung bei den Haarbasiszellen der verschiedenen Tarsenhaare und Borsten. Ueber den Inhalt dieser Vacuolen kann vorerst nichts ausgesagt werden, doch scheint es, in Analogie zu den Verhältnissen bei den Hypodermiszellen des Abdomens als sehr wahrscheinlich, daß sie in vivo mit Lipoiden resp. Lipoproteinen gefüllt sind.

Die Vacuolenbildung in den Hypodermiszellen konnte bei beiden untersuchten *R*-Stämmen gefunden werden, bei *R*-Stamm bei allen 10 untersuchten Fliegen, beim *RK*-Stamm deutlich bei 8 von 10 Fliegen, wobei bei zwei Fliegen die Vacuolenbildung nur schwach, aber doch sichtbar vorhanden war. Daraus darf wohl geschlossen werden, daß diese hypodermalen Vacuolen eine Eigentümlichkeit der *R*-Stämme darstellen und zum Wesen der Resistenz gehören.

Ueber die Bedeutung dieser Vacuolen kann man sich nachfolgendes Bild machen.

Die Gesamtcuticula ist auch noch nach ihrer Bildung von Porenkanälen durchzogen, die es den Epidermiszellen ermöglichen, die sich beim Gehen abnützende Epicuticula fortwährend zu erneuern. Es ist daher sehr wahrscheinlich, daß die lipoidhaltigen Inhaltsstoffe der Vacuolen in den Hypodermiszellen der *R*-Fliegen durch die Porenkanäle in die Cuticula, speziell in die Epicuticula abgegeben werden und damit auch der größere Lipoidgehalt der Epicuticula bei den *R*-Fliegen erklärt werden könnte. Allgemein erhält man aus den mikroskopischen Schnitten den Eindruck, daß die Hypodermiszellen der *R*-Fliegen viel aktiver sind als diejenigen der *S*-Fliegen.

Andererseits könnte die Bedeutung der hypodermalen Vacuolen darin bestehen, daß sie die durch die Cuticula eindringende lipophile DDT-Substanz abfangen und dadurch blockieren helfen. Die Lage der Vacuolen, der Cuticula angenähert oder gar angelagert, würde diese Funktion sehr begünstigen.

Nicht nur die Tarsen, sondern auch die Tibia und hie und da auch der Femur weisen in den Hypodermiszellen solche Vacuolenbildungen auf, hier besonders in den Basalzellen der Haare und Borsten. Wir werden zudem noch feststellen, daß

bei den *R*-Fliegen auch im übrigen Körperepithel solche vacuoligen Zellen vorkommen, die bei den *N*-Fliegen fehlen.

Die Nervenstränge der Tarsen (Abb. 4). Der Beinnerv, der in zwei Strängen bis in die Spitze des 5. Tarsengliedes über den latero-ventral gelegenen Sinneszellenpolstern verläuft, zeigt in den mikroskopischen Schnittbildern zwischen den *S*- und *R*-Fliegen keine Unterschiede. Ob solche durch spezielle Nervenfärbung gefunden werden können, bleibt noch zu untersuchen.

Der eine der Nervenstränge umschließt einen Tracheenstrang, während der andere frei davon ist.

In diesen beiden Nervensträngen konnte eine Struktur gefunden werden, die eventuell zur Erklärung des Autotremors der Fliegenextremitäten unter dem Einfluß der DDT-Substanz beiträgt. Es ist bekannt, daß Fliegen unter der Einwirkung von DDT-Substanz einen andauernd starken Beintremor aufweisen, der auch nach Amputation der Extremität bei denselben oft noch stundenlang weiterdauern kann. Diesen Autotremor kann man zudem auch erzeugen, wenn man isolierte Fliegenextremitäten Dämpfen von Benzol, Chlorbenzol etc. aussetzt, oder die Tarsen mit Diphenyl, DDT-Substanz und ähnlichen chlorierten Kohlenwasserstoffen bestreicht (WIESMANN, 1951; 1955b).

Es ist nun schwer verständlich, wie dieser Autotremor zustande kommt, denn normalerweise sind Beinbewegungen ohne Reflexbogen über ein Ganglion resp. ein motorisches Zentrum nicht möglich.

Bei der systematischen, genauen Durchsicht der Schnittbilder durch die Tarsen, besonders von Längsschnitten, konnte in jedem Tarsensegment im distalen Drittel der beiden Nervenstränge eine schwache, aber deutliche Verdickung gefunden werden, in der sich eine große Zelle befindet, die sich in vielen Beziehungen von den zahlreichen Neurilemzellen des Nervenstranges unterscheidet. Während die Neurilemzellkerne im Längsschnitt langgezogene Ellipsen darstellen, von einer Länge von 6–10 μ, ausgestattet mit viel basophilem Chromatin, ist dieser Einzelkern 40–45 μ lang und 10–12 μ breit und relativ chromatinarm. Er zeigt regelmäßig einen stark gefärbten Nucleolus und zudem kleinere, schwächer gefärbte Chromatinbrocken. Rings um den Zellkern ist ein mit Haematoxylin mehr oder weniger stark gefärbter Plasmahof zu finden, der sich deutlich von den nur schwach tingierten Nervenfasern abhebt.

Die ganze Struktur dieses Gebildes stimmt auffallend mit den motorischen Ganglienzellen im Bauchmark und Gehirn der Fliegen überein, sowohl was Größe als auch Form anbetrifft. Wie diese besitzt sie den als Nisslsche Substanz anzu sprechenden Plasmahof, einen großen Nucleolus und eine gut entwickelte Zellmembran.

Es darf daher wohl angenommen werden, daß in den beiden tarsalen Nervensträngen pro Tarse je 10 motorische Ganglienzellen sich vorfinden, die ihrer Aufgabe entsprechend Reize der tarsalen Sinnesorgane direkt zu den Extremitätenmuskeln übertragen, also einen vorgeschalteten kleinen Reflexbogen ermöglichen. Dieser kleine Reflexbogen dient möglicherweise dazu, Haltesignale zu übermitteln, in den Fällen, wo die Fliege mit den chemotaktischen Sinnesorganen ihrer Tarsen in einen Zuckertropfen etc. tritt und der Rüsselreflex erfolgt.

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Abb. 1. Tarse eines Mittelbeines von *Musca domestica*.

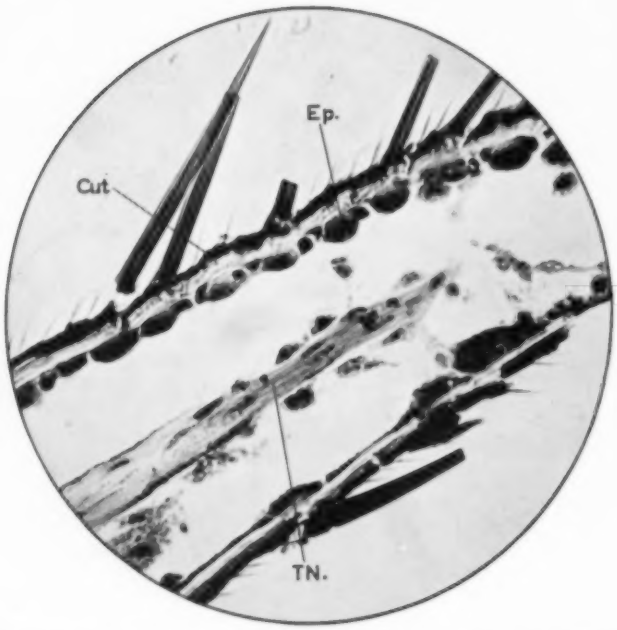

ABB. 2(a). Längsschnitt durch das 3. Tarsenglied einer sensiblen Fliege des Stammes *SN*. Vergr. 300×. *Ep:* Epithelzellen der Hypodermis. *Cut:* Cuticula. *Tn:* Tarsennerv.

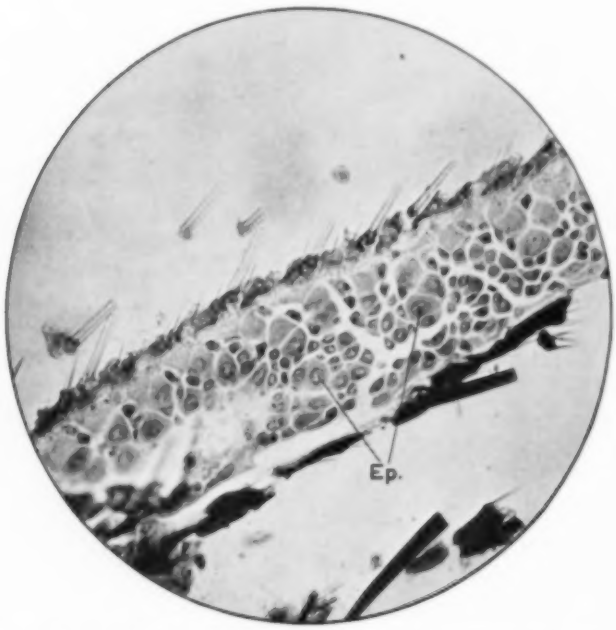

ABB. 2(b). Flächenschnitt durch das 3. Tarsenglied einer sensiblen Fliege des Stammes *SN*. Vergr. 300×. *Ep*: Epithelzellen der Hypodermis.

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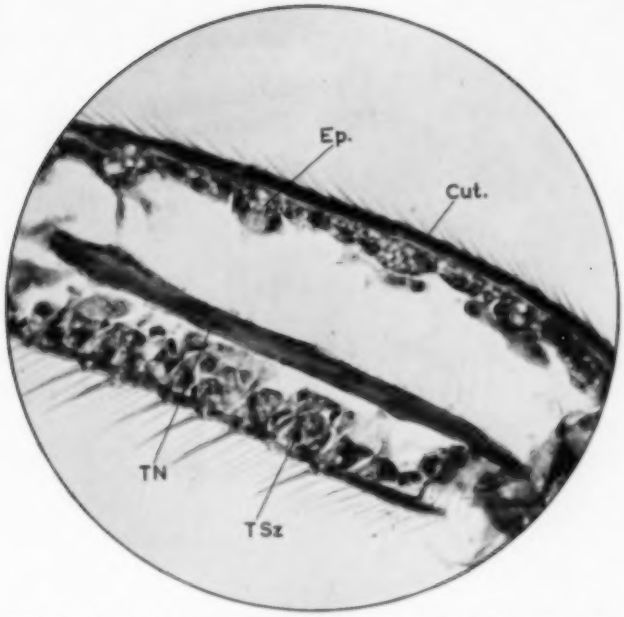

Abb. 3(a). Längsschnitt durch das 3. Tarsenglied einer resistenten Fliege des Stammes *R.* Vergr. 300×. *Ep:* Epithelzellen der Hypodermis mit Vacuolen. *Cut:* Cuticula. *Tn:* Tarsennerv. *TSz:* tarsale Sinneszellen.

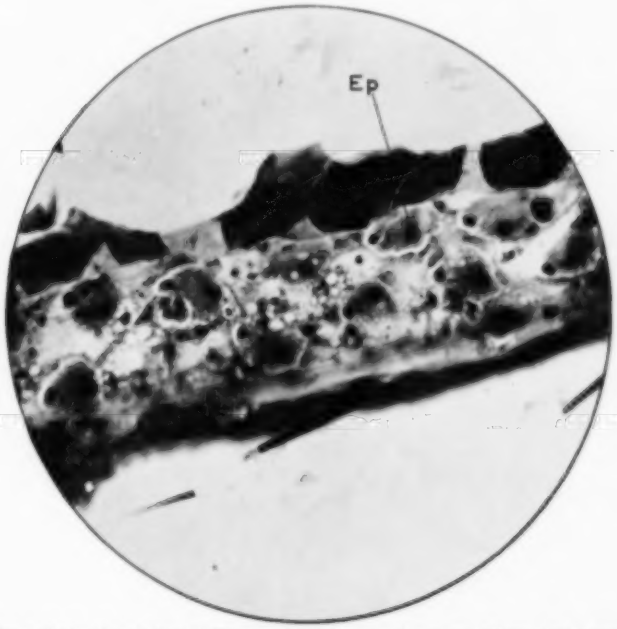

Abb. 3(b). Flächenschnitt durch das 3. Tarsenglied einer resistenten Fliege des Stammes *R.* Vergr. 300×. Das Plasma der Epithelzellen der Hypodermis (*Ep*) weist große und kleine Vacuolen auf.

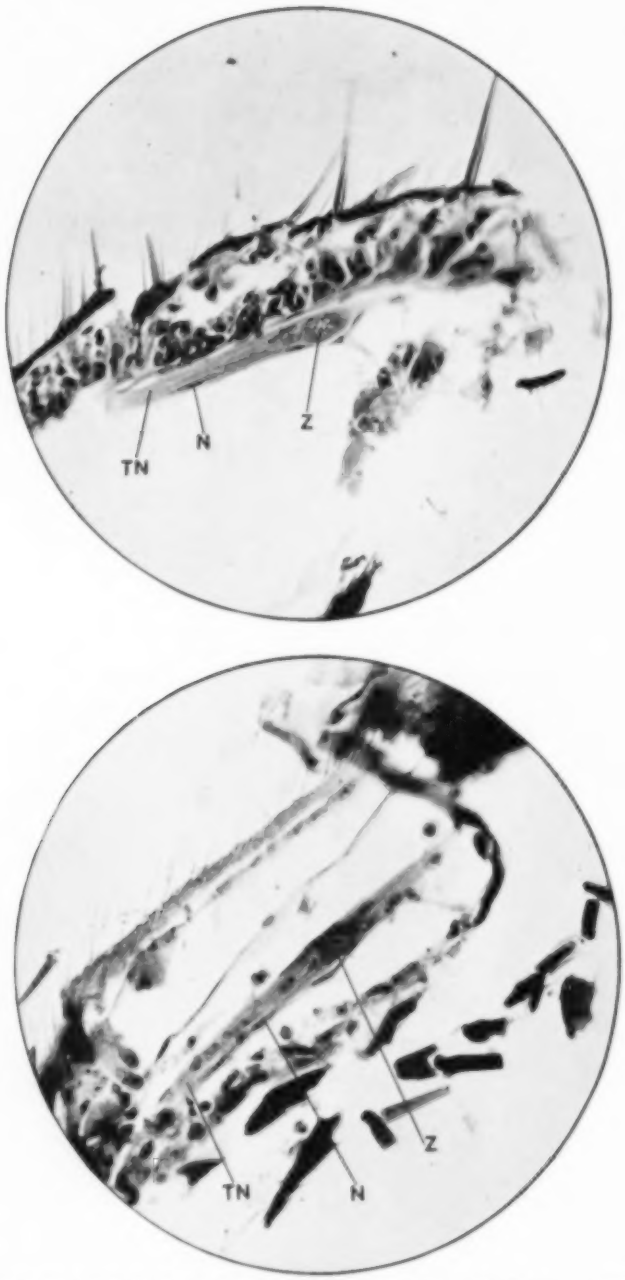

ABB. 4. Längsschnitt durch das 2. resp. 3. Tarsenglied einer Fliege des Stammes *SN*. Der Tarsennerv (*Tn*) zeigt eine schwache Verdickung, in der die als motorische Nervenzelle anzusprechende große Zelle (*Z*) sich befindet. *N* = Neurilemzellen.

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Abb. 5. Längsschnitt durch das 5. Tarsenglied einer sensiblen Fliege des Stammes *SN* in der Gegend der Tarsendrüsen *TD*.

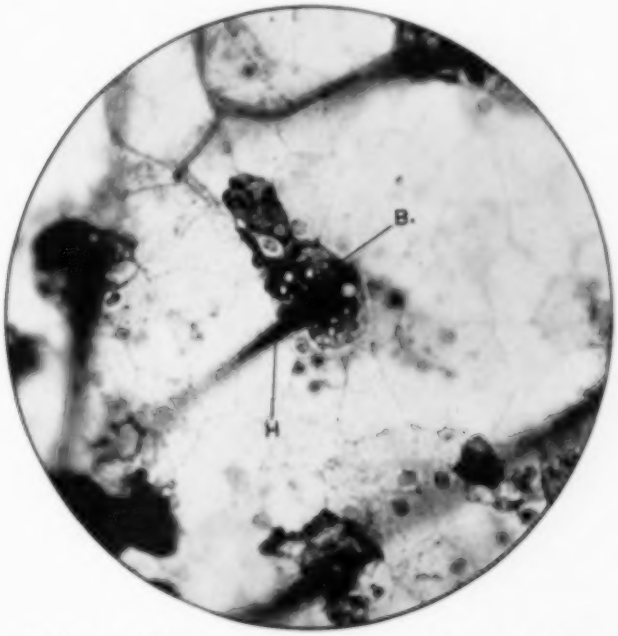

Abb. 6. Hypodermiszellen am Abdomen von *R*-Fliegen. Vergr. ca. 350×. Die Basalzellen (*B*) der Haare (*H*) sind mit Fett-Tröpfchen angefüllt.

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Des weitern kann dieser kleine, im Bein verlaufende Reflexbogen auch dafür verantwortlich sein, daß die amputierten oder isolierten Extremitäten unter dem Einfluß der DDT-Substanz einen Autotremor ausführen. Die gereizten Sinnesorgane geben die Reize an die in den Tarsennerven gelegenen motorischen Nervenzellen weiter, die dann die Beinmuskeln in Funktion setzen.

Tarsale Drüsen und ihre Ausscheidungen (Abb. 5)

Im 5. Tarsenglied jeder Extremität befindet sich eine große, gelappte Drüse, deren Exkrete in die Pulvillen und den Prätarsus abgegeben werden und das Haften der Fliegen auf den senkrechten, glatten Flächen ermöglichen. Diese Drüsen helfen sehr wahrscheinlich auch bei der Aufnahme und Resorption von Kontaktinsektiziden von einer behandelten Unterlage.

Es interessierte uns zu wissen, ob zwischen resistenten und sensiblen Fliegen Unterschiede in der Intensität der Funktion dieser Tarsendrüsen vorliegen, die unter Umständen mit der Insektizidresistenz im Zusammenhang stehen könnten.

Zur quantitativen Bestimmung der tarsalen Ausscheidungen brachte man einzelne *S*- und *R*-Fliegen auf Chromatographiepapier, resp. auf ganz saubere Objektträger und ließ die Tiere, in runden, niedrigen Glaskäfigen eingeschlossen, 24 Stunden auf der Fläche laufen. Nach dieser Zeit erkannte man auf dem Glas deutliche Laufspuren, hervorgerufen durch die Ausscheidungen der Tarsaldrüsen.

Ein Vergleich der Laufspuren von *S*- und *R*-Fliegen ergab, daß die Spuren der *S*-Fliegen deutlicher zu sehen waren als diejenigen der *R*-Fliegen. Diese Laufspuren liessen sich mit Fettfarben, wie Nilblausulfat, Fettschwarz Geigy, Sudan III bei den *S*-Fliegen 1–1½ mal stärker anfärben als diejenigen der *R*-Fliegen. Nach den erhaltenen Farbreaktionen handelt es sich bei den Ausscheidungen der Tarsendrüsen wahrscheinlich um Lipoide oder lipoidähnliche Substanzen. Faßbare histologische Unterschiede in der Struktur und Größe der Tarsendrüsen konnten zwischen *S*- und *R*-Fliegen nicht gefunden werden. Diese Unterschiede in der Sekretions- und Exkretionstätigkeit der Tarsendrüsen bei den *S*- und *R*-Fliegen müssen demzufolge rein physiologischer Art sein.

Die stärkere Lipoidausscheidung der Tarsendrüsen bei den *S*-Fliegen bedingt möglicherweise auch eine stärkere Resorption von DDT-Substanz durch die Tarsen als dies bei den *R*-Fliegen mit ihrer schwächeren Tarsendrüsentätigkeit der Fall ist. Einen Beweis für diese Annahme liefern frühere Untersuchungen (WIESMANN und REIFF, 1956). Es konnte gezeigt werden, daß die nach 30 Minuten tarsal aufgenommene Menge von DDT-Substanz bei den *S*-Fliegen mehr als doppelt so groß ist als bei den *R*-Fliegen. Dieser unterschiedliche Resorptionsmechanismus steuert sicherlich auch einen nicht unwichtigen Teil an das Resistenzgeschehen bei.

DIE HYPODERMISZELLEN DES ABDOMENS (Abb. 6)

Die Hypodermiszellen der Körperhaut der Imagines von *Musca domestica* können am besten an den Tergiten des Abdomens studiert werden, nachdem der

anhaftende Fettkörper sorgfältig wegpräpariert worden ist. Sehr instruktive Bilder erhält man durch Vitalfärbung der Tergitenkalotten mit Trypanblau nach Vonwiller. Nach einer Färbedauer von 1–3 Stunden und gründlichem Auswaschen in Aqua dest. können die Präparate in üblicher Weise in Canadabalsam eingeschlossen werden, wo sie sich, ohne zu verblassen, sehr gut halten.

Die Hypodermiszellen am Abdomen weisen linsenförmige Zellkerne auf. Sie sind ziemlich chromatinarm, und ihr zugehöriger Plasmaanteil ist durch deutlich anfärbbare Zellgrenzen ausgezeichnet (Abb. 6). Zwischen den *S*- und *R*-Fliegen lassen sich an diesen Zellen keine gesicherten Unterschiede feststellen. Dagegen sind an den Basalzellen der großen Haare und Borsten der Abdomenoberseite zwischen den *S*- und *R*-Fliegen Unterschiede vorhanden, die sicherlich mit der Resistenz als solcher gewisse Beziehungen aufweisen. Diese nierenförmigen, großen Basalzellen, von den übrigen Hypodermiszellen durch scharfe Zellgrenzen abgesetzt, weisen bei den *R*-Fliegen als Einschlüsse regelmäßig Fettröpfchen auf. Diese können durch Vitalfärbung mit Sudan III leicht angefärbt und dadurch sichtbar gemacht werden. Bei den *S*-Fliegen findet man an diesen Orten keine Fettröpfchen, oder in Einzelfällen vereinzelte kleine Fettkügelchen. Präparate, die über Xylol in Balsam eingeschlossen wurden, zeigen an den Stellen der Fetteinschlüsse leere Vacuolen. Die Bilder erinnern sehr stark an die Verhältnisse der vacuolisierten Hypodermiszellen der Tarsen.

Ueber die Bedeutung dieser Fettröpfchen in den Basalzellen der *R*-Fliegen im Resistenzgeschehen kann man vorerst nur Vermutungen anstellen. Man kann sich vorstellen, daß die DDT-Substanz, die durch die Haarpapillen als den Haupteintrittspforten in den Körper eindringt, in diesen Lipoiden gelöst wird, und daß dadurch eine gewisse Eindringungsbarriere entsteht.

Weiter sind sie möglicherweise auch an der Bildung der starken lipoidhaltigen Epicuticula der resistenten Fliegenkörper maßgebend beteiligt, indem sie, wie bei den Tarsen, in die Epicuticula abgeschieden werden.

Unter dem Einfluß der DDT-Substanz, d.h. bei Fliegen, die in eine 1%ige alkoholische Lösung von DDT-Substanz kurz eingetaucht wurden, scheinen sich die Fettröpfchen in den Haarbasiszellen, ungefähr 15–20 Minuten nach dem Eintauchen, deutlich zu vermehren, also eine Verbesserung der Eindringungsbarriere zu bilden.

Wenn auch bei Normalkontakt der Fliegen mit der DDT-Substanz, wie bereits angetönt, die Hauptmenge der Substanz durch die Tarsen aufgenommen wird, so erhält doch, meist innert kurzer Zeit, die ganze Körperoberfläche DDT-Substanz. *Musca* besitzt nämlich einen sehr stark ausgeprägten Putzreflex, der unter dem Einfluß der DDT-Substanz noch intensiviert wird, sodaß die an den Tarsen haftende Substanz über den ganzen Körper ausgebreitet wird. So können diese, den *S*-Fliegen fehlenden Lipoideinschlüsse in der Abdomenhaut zur Inaktivierung der DDT-Substanz bei den *R*-Fliegen von Bedeutung werden.

Diese Fetteinschlüsse in den basalen Haarzellen wurden bei unseren beiden *R*-Stämmen regelmäßig gefunden, sodaß angenommen werden kann, daß diese Einschlüsse zum Wesen der Resistenz gehören.

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ZUSAMMENFASSUNG UND DISKUSSION

Im Zusammenhang mit Untersuchungen über die auf Selektion beruhende Resistenz von *Musca domestica* L. gegenüber dem als Kontaktinsektizid wirksamen Dichlordiphenyltrichloräthan (DDT-Substanz) wurde auch vergleichend die Histologie der Aufnahme- und Erfolgsorgane der DDT-Substanz bei 2 resistenten (*R* und *RK*) und 2 sensiblen Stämmen (*SN* und *SK*) der Stubenfliege untersucht, nämlich der Tarsen und des Körperintegumentes.

In der Cuticula der Tarsen konnten lichtoptisch keine Unterschiede festgestellt werden, dagegen kamen, durch die Behandlung derselben mit verschiedenen Agenzien, deutliche Unterschiede im Lipoid- und Lipoproteingehalt der Epicuticula und im Inkrustenanteil (Proteide und Lipoproteide) zum Vorschein. Die Epicuticula der *R*-Fliegen besitzt 30–40% mehr Lipoide und die Gesamtcuticula ca. 1 mal mehr Inkrusten. Der erhöhte Gehalt der Tarsencuticula an Lipoiden, Lipoproteiden und Proteiden steht wahrscheinlich im Zusammenhang mit der stärkeren Blockierungsfähigkeit der *R*-Tarsen gegenüber der lipophilen, enzymatisch nicht abgebauten DDT-Substanz.

Auch in der Hypodermiszellschicht der Tarsen sind zwischen den *S*- und *R*-Fliegen Unterschiede gefunden worden, die mit der Resistenz zusammenzuhängen scheinen. Im Gegensatz zu den *S*-Fliegen weist der Protoplasmakörper der Hypodermiszellen größere und kleinere Vacuolen auf, die wahrscheinlich mit Lipoiden erfüllt sind. Dieselben haben möglicherweise eine doppelte Bedeutung. Sie bedingen einerseits den hohen Lipoidgehalt der Epicuticula bei den *R*-Fliegen und können andererseits die durch die Tarsen eindringende DDT-Substanz direkt blockieren helfen.

Im, in zwei Strängen durch den Tarsus verlaufenden Beinnerv wurden vorerst keine Unterschiede zwischen den *S*- und *R*-Fliegen festgestellt. Dagegen konnten in jedem Tarsensegment, im distalen Drittel der beiden Nervenstränge, schwache Verdickungen mit je einer großen Nervenzelle gefunden werden, deren Struktur mit derjenigen der motorischen Ganglien des Gehirns und des Bauchmarkes übereinstimmt. Demnach darf angenommen werden, daß in den beiden tarsalen Nervensträngen je 10 motorische Ganglienzellen sich vorfinden, die Reize der tarsalen Sinnesorgane direkt zu den Extremitätenmuskeln übertragen, also einen kleinen, vorgeschalteten Reflexbogen ermöglichen. Durch diesen Befund kann jetzt auch der Autotremor isolierter Extremitäten unter Einwirkung von DDT-Substanz besser verstanden werden.

Die im 5. Tarsenglied gelegene, gelappte Tarsendrüse scheidet bei den *S*-Fliegen 1–1½ mal mehr lipoidähnliche Substanz aus als bei den *R*-Fliegen, worauf die größere Resorption von DDT-Substanz bei den *S*-Fliegen zu beruhen scheint.

Im Unterschied zu den *S*-Fliegen weisen bei den *R*-Fliegen die epidermalen Haarbasiszellen des Abdomens als Fettröpfchen erkennbare Einschlüsse auf, die unter der Einwirkung von DDT-Substanz auf die Fliege noch vermehrt werden. Diese Einschlüsse tragen einerseits zum allgemein deutlich erhöhten Totallipoidgehalt der *R*-Fliegen bei und andererseits bedingen sie eine gewisse Eindringungsbarriere für die DDT-Substanz, indem die durch die Haarpapillen als

Haupteintrittspforten eindringende Substanz in den Lipoiden gelöst und dadurch blockiert wird.

Alle diese physiologischen und histologischen Unterschiede scheinen mit der Resistenz in direkter Beziehung zu stehen, indem sie zu einer Blockierung der im *R*-Fliegenkörper verbleibenden, enzymatisch nicht abgebauten DDT-Substanz direkt oder indirekt beitragen und ihre Toleranz ermöglichen.

Die *R*-Fliegen können demnach in verschiedenen Beziehungen von den sensiblen unterschieden werden, sowohl durch physiologische Eigenschaften als auch durch Strukturunterschiede, die ebenfalls mit der Resistenz in Zusammenhang stehen.

Wir müssen uns darüber im klaren sein, daß das ganze Resistenzgeschehen sehr komplex aus verschiedenen Faktoren zusammengesetzt ist, wobei die Einzelfaktoren bei den verschiedenen Stämmen sehr verschiedenes Gewicht haben können. Daher ist auch zu vermuten, daß die hier mitgeteilten physiologischen und histologischen Unterschiede zwischen unseren *S*- und *R*-Stämmen nicht unbedingt bei allen Stämmen in gleicher Stärke ausgebildet sein müssen, und bei anderen Stämmen unter Umständen noch andere, hier nicht vorhandene Unterschiede vorliegen können, die aber für die betreffenden Stämme auch wieder zur Resistenz beitragen.

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SUMMARY

The comparative aspects of the tarsi and cuticle of two resistant strains (*R* and *RK*) and two susceptible strains (*SN* and *SK*) of the housefly have been examined.

No difference was noted between the two strains in the cuticle of the tarsi on optical examination. But treatment of the tarsi with various reagents made obvious very definite differences in the lipoid and lipoprotein content of the epicuticle and in the incrustations (proteins and lipoproteins). The epicuticle of the resistant flies has 30–40% more lipoids than that of the susceptible flies, and the total cuticle has approximately twice as many incrustations. The stronger blocking of the resistant tarsi to the active ingredient of DDT, which is lipophilous and not metabolized by enzymes, is probably related to the higher content of lipoid, lipoprotein, and proteins in the tarsal cuticle.

Differences were also noted between the tarsal epidermis of susceptible and resistant flies. The protoplasm of the epidermal cells in resistant flies shows small and large vacuoles probably containing lipoids. These vacuoles may have a two-fold function: they are responsible for the high lipoid content of the epicuticle in the resistant flies and may, furthermore, assist in blocking the active ingredient of DDT penetrating through the tarsi.

No difference has yet been found between the tarsal nerve of the sensitive and resistant flies. This nerve is formed of two cords in the distal third of each tarsal segment. A slight thickening has been found on both nerve cords. Its structure is the same as that of the motor ganglia of the brain and of the ventral nervous chain. It can therefore be assumed that ten motor ganglia are present in both tarsal nerve cords and that they transmit the stimuli of the tarsal sense organs directly to the muscles of the extremities. This arrangement forms a small reflex arc, and in this way may be explained the independent tremor of isolated extremities

after the action of the active ingredient of DDT. Another difference has been found in the gland present in the fifth tarsal segment. In the susceptible strain this gland secretes 100–150% more lipoid-like substance than that of the resistant strain, and this seems to be responsible for the higher resorption of the active ingredient of DDT in susceptible flies.

Unlike the susceptible flies, the resistant flies have inclusions in the epidermal cells at the base of setae on the abdominal wall. These inclusions are small fatty drops, and, under the action of the active ingredient of DDT, they increase in number. These inclusions contribute to the higher total lipoid content of the resistant flies. They also act as a barrier against the penetration of the active ingredient of DDT because the substance entering through the papillae of the setae is dissolved in the lipoids and thus blocked.

All of these physiological and histological differences seem to be related to resistance in that they contribute directly or indirectly to the blocking of the unmetabolized active ingredient of DDT and thus enhance the tolerance of the resistant flies to the insecticide.

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NUTRITIONAL REQUIREMENTS OF *AEDES AEGYPTI* L.*

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Abstract—Larvae of *Aedes aegypti* can be reared on a chemically defined diet consisting of glucose, salt mixture, lipids, RNA, 17 amino acids, glutathione, and 12 vitamins, provided the osmotic pressure of the diet does not exceed △0·4°C. L-Valine, L-leucine, L-*iso*leucine, L-phenylalanine, L-histidine, L-arginine, L-tryptophane, L-threonine, L-methionine, and L-lysine are absolutely essential for larval growth. L-cystine is essential for pupation and L-proline, L-hydroxyproline, and L-serine are required for normal growth and development. Glycine, L-tyrosine, and L-alanine are not required. Thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotin are essential for larval growth. Folic acid and pyridoxine are definitely required for pupation; vitamin B_T and choline chloride are also required for normal growth and development. The absence of vitamin B_{12} delays pupation and *p*-aminobenzoic acid delays ecdysis, and the absence of *i*-inositol is entirely without effect. Glutathione is essential for normal growth and development, and it cannot be replaced by its component amino acids. Glucose, salt mixture, lipids, and RNA are also essential for normal growth and development.

A mixture of amino acids is a sufficient diet for an adult female to mature viable eggs. The essential amino acids for this function are: valine, leucine, *iso*leucine, phenylalanine, histidine, arginine, tryptophane, threonine, methionine, and lysine. These are also the ten indispensable amino acids for larval nutrition.

INTRODUCTION

THE premiss of this investigation is that the nutritional state of adult mosquitoes required for production of viable eggs is derived not only from food taken after emergence but also from the nutrition during the larval stage. It should therefore be possible to determine under aseptic conditions the food requirements of *Aedes aegypti* in terms of pure nutritional compounds for the completion of a generation from egg to egg.

The study of larval nutrition of mosquitoes was inaugurated by TRAGER (1935a, b), who was able to rear the larvae of *Aedes aegypti* to the adult stage under aseptic conditions on a suspension of dried whole yeast powder, provided it was supplemented by liver extract. He also demonstrated that the larvae could utilize solutes as well as particulate food materials. Subsequently, SUBBAROW and TRAGER (1940) demonstrated that the addition of riboflavin and ribonucleic acid (RNA) to this diet increased the growth rate. FROST, HERMS and HOSKINS (1936) were able to rear sterile larvae of *Theobaldia incidens* either on brewer's yeast powder alone or

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on casein that was not vitamin-free and was supplemented by inorganic salts. De Meillon, Golberg and Lavoipierre (1945) then showed that autoclaved fresh whole yeast suspended in 0·01 N $CaCl_2$ was adequate for the larval development of *A. aegypti*.

Golberg, De Meillon and Lavoipierre (1945) proceeded to investigate the nutritional role of individual vitamins by using a basal diet consisting of the insoluble residue of yeast, supplemented by glucose, RNA, glutathione, 9 vitamins, and inorganic salts. In this way they were able to show that thiamine, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid were essential for full growth of the larvae, and that folic acid was required for pupation. Two workers succeeded in replacing the insoluble residues of yeast by some form of casein with appropriate accessory factors: Trager (1948) reared *A. aegypti* larvae on vitamin-free casein supplemented with 6 vitamins mentioned above, together with L-cystine, pyridoxamine, niacinamide, and cholesterol; and Lichtenstein (1948) reared *Culex molestus* on casein hydrolysate supplemented with vitamins, as well as glucose and ascorbic acid.

In a subsequent paper Golberg and De Meillon (1948b) reported their investigations of the nutritional role of amino acids. In these experiments the basic diet consisted of yeast autolysate supplemented with glucose, lipids, vitamins, and inorganic salts; 17 amino acids were then added and the effect of subtracting each one was investigated. In this manner 11 amino acids were found to be essential for larval growth and development, namely, glycine, L-leucine, DL-*iso*leucine, L-histidine, L-arginine, L-tryptophane, DL-threonine, DL-methionine, DL-phenylalanine, and L-tyrosine. In addition, L-cystine was necessary for satisfactory emergence. That Golberg and De Meillon succeeded in getting significant results of this nature is quite remarkable because the yeast autolysate in their basic diet already contained a high amount of all the amino acids. The medium in which the larvae grew contained 20 g of yeast autolysate (dry weight) per litre of aqueous solvent, while the total amount of all the amino acids added was only 10 g/l.

Although the nutrition during the larval stage is sufficient to ensure emergence of the adult mosquito, it is usually insufficient to allow maturation of the ovaries of the female, which therefore requires a meal of vertebrate blood. Certain autogenous species of mosquitoes such as *Culex pipiens* (Huff, 1929; Richards, 1941), *Aedes scutellaris* (Ken, 1917), and *Aedes atropalpus* (Trambley, 1946) can lay their initial batch of eggs without a blood meal. Oviposition without feeding on blood is also reported by Marshall and Staley (1936) for *Theobaldia subochrea* and by Woodhill (1936) for *Aedes concolor*. In the study of the autogenous species *Aedes communis* Hocking (1954) found that the flight muscles of the female histolysed and provided sufficient protein to mature its eggs; he also observed a similar histolysis in the non-biting mosquito *Mochlonyx culiciformis*. On the other hand, Beckel (1954) was unable to find any histolysis in the flight muscles of *A. communis*; instead he suggested that the protein was supplied by the fat body. It had already been shown histochemically by Bettini (1947) that the autogenous

Culex pipiens (*molestus*) differs from anautogenous mosquitoes by the deposition of protein in the fat body and its subsequent mobilization during ovarian development.

The majority of species of mosquitoes are anautogenous in that the females require a blood meal to mature their eggs. A typical example is *A. aegypti*. MARCHOUX and SIMOND (1906) found that only the whole blood would induce oviposition, whereas serum, erythrocytes, or clotted blood were ineffective; similar results with human blood were obtained by GORDON (1922). But WOKE (1937) obtained oviposition in this species with rabbit serum, plasma, and haemoglobin as well as with defibrinated, heparinized, and decalcified blood. Results reported by MER (1936) and YOELI and MER (1938) with *Anopheles elutus* indicated that neither haemoglobin nor serum was alone capable of inducing oviposition, but that haemoglobin induced the initial development of the ovaries and serum can then complete the maturation of the eggs. GREENBERG (1951) then showed that whereas the blood-cells were important to promote feeding, it was the protein in the serum which allowed *A. aegypti* to mature its eggs. By adding various proteins to a suspension of sheep erythrocytes, which alone induce only slight egg development, he was able to obtain considerable oviposition. He succeeded in substituting *iso*leucine for the protein and obtained oviposition. On the other hand, substitution of the inorganic salts, vitamins, glucose, and fat for protein did not promote egg development.

In these experiments the mosquitoes fed on the materials through a stretched membrane of animal skin, and they required the stimulus of some haematological material to pierce this membrane. The study of chemically defined nutrients requires methods other than membrane feeding. MACGREGOR and LEE (1929) were the first to succeed in feeding *A. aegypti*, *C. pipiens*, and *Anopheles maculipennis* on a cotton pad soaked in citrated blood to which a little honey had been added. MCLINTOCK (1952) used this method for adult *Culiseta inornata*, substituting sucrose for honey. A mixture of one part honey and nine parts citrated beef blood exposed on a cotton pad warmed to 33–35°C is now successfully used as a standard procedure for feeding *A. aegypti* and *Anopheles quadrimaculatus* (LEA *et al.*, 1955). Using these techniques, LEA *et al.* (1955) found that *A. aegypti* and *A. quadrimaculatus* can develop and lay viable eggs after feeding on skimmed milk and honey. Adults of *A. quadrimaculatus* also laid viable eggs when fed on honey added to a mixture of proteose-peptone, liver concentrate, and casein hydrolysate. DIMOND *et al.* (1955) then showed that *A. aegypti* could mature eggs on a mixture of 18 amino acids in a 10 per cent solution of honey in water administered daily on a cotton pad from the time of emergence. They also found that honey can be successfully replaced by a combination of fructose and glucose. The addition of an inorganic salt mixture resulted in a moderate increase in egg production. Subsequently, LEA *et al.* (1956) showed that only 10 amino acids were necessary, namely, L-arginine, DL-*iso*leucine, L-leucine, DL-phenylalanine, DL-threonine, L-tryptophane, DL-valine, L-histidine, DL-methionine, and L-cystine.

It will be noted that the amino acids found by LEA *et al.* (1956) to be essential for egg production in adults correspond with the amino acids reported by GOLBERG

and DE MEILLON (1948b) to be essential for development of larvae. The adults of LEA *et al.* (1956) were derived from larvae fed on stock insectary materials under conditions that were not aseptic; the larvae of GOLBERG and DE MEILLON were never followed through the adult stage and oviposition. Therefore the experiments to be reported in this paper deal with the nutritional requirements of both the larvae and adults, reared with aseptic precautions, so that the nutrition from hatching of egg to deposition of the egg of the next generation is fully characterized.

MATERIAL AND METHODS

Larvae of *Aedes aegypti* were fed on synthetic diets in Pyrex test-tubes, and aseptic precautions were taken. Contaminated samples were quickly evident by the development of turbidity in the otherwise clear yellow solution and by a rapid increase in larval growth; these were immediately discarded. When the clear samples were tested by placing them on yeast-dextrose agar they proved to be sterile in all 10 samples taken for testing. The adults which had been reared under aseptic precautions were fed on sterile diets on cotton pads, but their cages were not sterile.

All procedures involving transfer of eggs, larvae, adults, or diets were carried out in a transfer chamber similar to that described by HOUSE and PATTON (1949), although on simpler lines. Its dimensions were 36 in. long, 24 in. wide, and 30 in. high. The front window was hinged at the top and there was a small door on the left side. The chamber contained an ultra-violet lamp for sterilizing the air.

Sterile larvae were obtained by treating the eggs taken from the insectary stock of *A. aegypti*. The eggs were laid on paper towelling partially immersed in laundry tubs. They were then conditioned by holding them at 28°C and 70 per cent R.H. for 3 days after oviposition (TREMBLEY, 1955) and thereafter were held at normal room temperature and humidity (25 per cent R.H.) until used. The conditioned eggs were separated from the paper and placed in White's solution for 15 min (WHITE, 1931) for sterilization. At the end of this time the sterilizing fluid was removed by filtration through sterilized filter paper inside the transfer chamber, and the eggs were washed several times with sterile distilled water. These sterilized eggs were washed down into another tube by making a hole in the bottom of the filter paper and rinsing them with a 0·05 per cent solution of salt mixture MD No. 185. Then these eggs were placed in an incubator at 30°C, and the larvae hatched within 1 hr. The larvae were separated by filtration, washed with sterilized distilled water, and were pipetted out into a Petri dish containing sterilized distilled water. The aseptic larvae thus obtained were inoculated into the diet under test before they were 2 hr old.

The diet originally used by GOLBERG, DE MEILLON and LAVOIPIERRE (1945), which contained a high proportion of the insoluble residue of yeast, was taken as the starting-point (Table 1). Initial efforts were concerned with substituting lipids, casein, and certain vitamins for the insoluble residue of yeast (Table 2); this necessitated preparing the diet in two separate parts. The solid fraction, originally consisting of the insoluble residue of yeast, consisted of lipids adsorbed on to the

insoluble casein employed. This was prepared by dissolving 0·25 g of lipid mixture in redistilled diethyl ether and adding it to 10 g of vitamin-free casein. The second part, the liquid fraction, contained all other ingredients of the diet. The required quantities of amino acids, vitamins, glucose, inorganic salts, and RNA were carefully weighed and transferred to a 250 ml flask. RNA and tyrosine were dissolved separately in minimum quantities of 0·1 N NaOH, cystine in 1·0 N HCl, folic acid in 1 per cent NH_4 OH, phenylalanine, and biotin in hot water. The solution was adjusted to pH 6·6 before being sterilized.

TABLE 1—ORIGINAL DIET EMPLOYED FOR NUTRITIONAL STUDY OF LARVAE
Based on the diets used by GOLBERG, DE MEILLON and LAVOIPIERRE (1945)

		g/l.
Glucose		10·0
Insoluble residue of yeast		6·25
RNA (Ribonucleic acid)		0·50
Vitamins		0·115
	mg/l.	
Thiamine	10	
Riboflavin	20	
Pyridoxine	10	
Ca Pantothenate	10	
Nicotinic acid	10	
Folic acid	30	
Choline Cl.	25	
Inorganic salts (MD No. 185)		1·50

TABLE 2—COMPOUNDS SUBSTITUTED FOR THE INSOLUBLE RESIDUE OF YEAST IN THE ORIGINAL DIET

		g/l.
Lipids*		0·156
	mg/l.	
Lecithin	78	
Cephalin	39	
Cholesterol	39	
Casein (vitamin-free)		6·25
Vitamins		
	mg/l.	
Biotin	0·5	
B_{12}	0·05	
B_T	4·0	
Glutathione	50·0	

* Adsorbed on to casein at the rate of 1 part to 40.

The liquid fraction of the diet was sterilized by suction filtration through a Seitz filter, and was then distributed to the sterilized test-tubes by means of a special dispensing flask. Suction was applied to the upper opening of the flask and

the diet was distributed to the test-tubes by the lower opening. The solid fraction was distributed to 18 × 120 mm test-tubes, 50 mg to each tube, and these were stoppered with cotton-wool and autoclaved at 18 lb/in² for 1 hr. The dispensing flask and ancillary glassware were autoclaved at the same time. The transfer chamber was sterilized by burning the ultra-violet lamp for 1 hr and spraying with White's solution, and 8 cm³ of the liquid fraction was transferred to each test-tube inside the chamber. One larva was then inoculated into each tube, and the cotton-wool plug was replaced. The test-tubes were placed in an incubator at 30°C, and observations were made every morning and evening. The stage of development of each larva was recorded, the exuviae remaining in the tube serving as a guide; and the length of time spent in each instar was thus computed. For each diet tested, the number of individuals completing each instar and the average duration of each instar with its standard error were computed and tabulated.

Two classes of diets were employed. In the first class (Tables 1 and 2) the solid fraction consisted either of the insoluble residue of yeast or of lipid mixture adsorbed on to vitamin-free casein. The insoluble residue of yeast was prepared by boiling brewer's yeast powder with 4 volumes of water and a few drops of capryl alcohol for 15 min and filtering. The entire process was repeated 3 or 4 times. The final residue was then freed of the last traces of soluble material by extracting continuously with boiling water in a Soxhlet apparatus. It was dried in a vacuum, ground in a meat grinder, and passed through an 80 mesh sieve. The proportions of materials used in liquid fraction are shown in Tables 1 and 2.

In the second class, i.e. the chemically defined diet (Table 3), the solid fraction consisted of 1 part lipid mixture adsorbed on to 40 parts of finely-powdered cellulose and the amino acid mixture was added in the liquid fraction. The proportions of amino acids were based on those present in casein, except that glutamic acid and aspartic acid were omitted and only the naturally occurring L-isomers were used. The proportions of vitamins in the diet were based on those employed by House (1954b).

The adult females were obtained by rearing larvae on the amino acid diet (Table 3) with sterile precautions. The emerging adults were removed by suction in a stream of sterilized air to preclude contaminating the remaining culture. To ensure the sterility of the air stream, a train consisting of 2 wash-bottles containing 0·05 per cent $HgCl_2$, 1 wash-bottle containing distilled water, and a $CaCl_2$ U-tube was introduced into the flask intake. The intake extended as a long tube almost reaching the surface of the culture liquid: by another entrance this tube had been used to admit the liquid fraction of the diet and the sterile larvae. The entire apparatus was sterilized before adding the larvae and the liquid fraction.

The adults which emerged from the sterile diet were distributed, in lots of 20 females plus 10 males, to cages consisting of 7 in. high inverted lantern globes, screened at the top and standing on moistened filter paper in a Petri dish. These cages were thoroughly clean but not sterile. The adult diets were administered in 2 cm³ portions on 2 cm² pads of sterilized absorbent cotton-wool, a fresh pad being introduced every day for 6 days. The cages were kept in an incubator at

30°C and 70 per cent R.H., which had been thoroughly sprayed with White's solution as an antiseptic measure. When oviposition occurred, it usually took place

TABLE 3—CHEMICALLY DEFINED DIET FOR LARVAE OF *Aedes aegypti*

		g/l.
Glucose		5·00
Lipids*		0·156
	mg/l.	
Lecithin	78	
Cephalin	39	
Cholesterol	39	
Amino acids		11·64
	g/l.	
L-Leucine	1·00	
L-*Iso*leucine	1·12	
L-Threonine	0·75	
L-Valine	1·20	
L-Phenylalanine	1·00	
L-Lysine (Mono-HCl)	0·66	
L-Arginine (Mono-HCl)	0·39	
L-Histidine (Mono-HCl)	0·25	
L-Tryptophane	0·36	
L-Methionine	0·70	
L-Cystine	0·13	
L-Proline	0·75	
L-Hydroxyproline	0·18	
L-Serine	1·40	
Glycine	0·25	
L-Alanine	1·00	
L-Tyrosine	0·50	
RNA		1·00
Vitamins		0·65
	mg/l.	
Thiamine	5·00	
Riboflavin	20·00	
Pyridoxine	15·00	
Ca Pantothenate	15·00	
Nicotinic acid	15·00	
Folic acid	50·00	
Choline Cl.	200·00	
Biotin	0·50	
B_T	4·00	
Glutathione	200·00	
B_{12}	0·05	
p-Aminobenzoic acid	25·00	
i-Inositol	100·00	
Inorganic salts (MD No. 185)		1·00

* Adsorbed on to cellulose at the rate of 1 part to 40.

between the third and sixth days. The eggs were collected and counted on the seventh day. They were then tested for viability by treatment with MD No. 185 hatching solution.

RESULTS

The larval diet employed by GOLBERG, DE MEILLON and LAVOIPIERRE (1945), based on the insoluble residue of yeast (Table 1), was found to allow development to emergence of the adult in an average of 8·6 days. It was also found that removal of RNA did not significantly decrease in the growth rate, presumably since it was present in the insoluble residue of yeast. But the removal of glucose resulted in only 40 per cent reaching the adult stage, while 50 per cent remained in the first larval stage for the entire 2 weeks' period of observation. This type of developmental arrest was more characteristic of inadequate diets than was mortality.

Then vitamin-free casein was substituted for the insoluble residue of yeast and supplemented by lipid mixture adsorbed on to it. This basal diet was then successively supplemented by cumulative addition of biotin, vitamin B_{12}, vitamin B_T (carnitine), and glutathione. The results are shown in Table 4. It will be seen

TABLE 4—EFFECT OF REPLACING THE INSOLUBLE RESIDUE OF YEAST WITH CASEIN, LIPIDS AND CERTAIN VITAMINS

	No. of larvae		I	II	III	IV	Pupa
Original diet as per Table 1	39	No. completing instar	38	37	37	37	37
		days to complete instar	1·6 ± 0·01	1·2 ± 0·01	1·2 ± 0·01	2·4 ± 0·03	2·0 ± 0·01
Insoluble residue of yeast replaced by casein (vitamin-free) and lipids as per Table 2, with the following vitamins :							
Biotin	20	No. completing instar	19	19	17	0	—
		days to complete instar	2·5 ± 0·18	1·7 ± 0·18	1·0 ± 0·04	—	—
Biotin + B_{12}	20	No. completing instar	20	20	17	0	—
		days to complete instar	2·9 ± 0·06	2·0 ± 0·05	2·4 ± 0·29	—	—
Biotin + B_{12} + B_T	20	No. completing instar	20	20	20	9	1
		days to complete instar	1·8 ± 0·06	2·2 ± 0·05	1·7 ± 0·05	3·3 ± 0·07	1·5 ± 0·00
Biotin + B_{12} + B_T + glutathione	40	No. completing instar	39	36	36	33	24
		days to complete instar	1·7 ± 0·06	1·5 ± 0·10	1·5 ± 0·05	3·0 ± 0·07	2·0 ± 0·00

that the basal diet and biotin succeeded in taking 90 per cent of the larvae to the fourth instar. The addition of vitamin B_{12} did not improve the diet, but, when vitamin B_T was added, 45 per cent of the larvae pupated and one adult emerged. When glutathione was added to these three supplementary substances, 60 per cent of the larvae reached the adult stage in an average time of 9·3 days. It must also be noted that when glutathione was not added but instead the concentration of the diet containing biotin, B_{12}, and B_T was doubled, 55 per cent reached the adult stage in an average of 12·7 days: presumably the increase in casein concentration supplied the additional SH groups otherwise afforded by glutathione.

With the successful elimination of the insoluble residue of yeast by means of supplementing vitamin-free casein with the above four accessory compounds, it was now possible to attempt replacing the casein by amino acid mixtures. In these first attempts the diet employed was that listed in Table 3. The amino acids were in a different proportion (Mixture *D* of GOLBERG and DE MEILLON, 1948b)

and they were not exclusively L-isomers. When added to the liquid fraction of glucose, inorganic salts, RNA, and 8 vitamins (the solid fraction containing lipids adsorbed on cellulose) the amino acid mixture failed to take the larvae beyond the second instar, nor did the addition of B_{12}, B_T, and glutathione improve the situation. When the amino acid mixture was coated with lipids and added as the solid fraction, most larvae reached the third instar and 10 per cent reached the fourth instar. Although the amino acid mixture sedimented to the bottom of the tube, its availability became reduced by the formation of a gelatinous film over it.

TABLE 5—REPLACEMENT OF CASEIN BY AMINO ACIDS, AND THE EFFECT OF THEIR CONCENTRATION ON DEVELOPMENT

Conc. (g/l.)	Δ (°C)	No. of larvae		I	II	III	IV	Pupa
2·0	0·15	20	No. to instar	0	—	—	—	—
			days in instar	—	—	—	—	—
4·0	0·23	20	No. to instar	6	0	—	—	—
			days in instar	13·9 ± 0·14	—	—	—	—
8·0	0·29	20	No. to instar	19	14	3	0	—
			days in instar	8·5 ± 0·80	6·4 ± 0·48	6·0 ± 0·13	—	—
11·64	0·39	20	No. to instar	20	20	20	16	16
			days in instar	2·9 ± 0·04	2·6 ± 0·05	3·0 ± 0·22	5·6 ± 0·35	2·0 ± 0·00
16·0	0·44	20	No. to instar	12	3	0	—	—
			days in instar	7·0 ± 0·52	10·0 ± 0·47	—	—	—
22·0	0·52	20	No. to instar	0	—	—	—	—
			days in instar	—	—	—	—	—

These experiments suggested that the reason for the failure of the soluble amino acids to replace insoluble casein as a suitable diet was their high osmotic pressure. Therefore, the osmotic limits of the diet for mosquito larvae were first investigated by adding various concentrations of glucose to the original diet (Table 1) containing the insoluble residue of yeast, inorganic salts, RNA, and 7 vitamins. The osmotic activity of these diets, which contained from 0·5 to 8·0 per cent glucose, was measured as the freezing-point depression from Δ0·12 to Δ0·98°C by Beckmann's cryoscopic method. It was found that the larvae could complete their development at osmotic pressures up to Δ0·37°C; at concentrations of Δ0·45°C and above they did not grow at all. This experiment could now be performed with amino acids as the principal osmotic source, using the diet listed in Table 3 and varying the concentration of amino acids to give osmotic equivalents between Δ0·15 and Δ0·52°C. The results (Table 5) show that optimum growth occurs at Δ0·39°C: greater concentrations inhibit development, and lower

concentrations contain insufficient dietary amino acids to allow full growth of the larvae.

The concentration of amino acids giving Δ0·39°C, which was 11·64 g/l., was taken as the basis for the completely-defined diet. It contained the following: 17 amino acids, 3 lipids, 8 inorganic ions, glucose, RNA, and 13 vitamins. The exact concentrations of each component of this diet are listed in Table 3. This diet was employed to rear the sterile adults used in the oviposition experiments. In all, 204 newly hatched larvae were inoculated singly on this diet. Of these, 72 per cent reached the adult stage in an average of 17·3 days. This performance may be compared with that obtained on yeast autolysate, insoluble residue of yeast, and vitamin-free casein, as follows:

Source of protein	Per cent adults	Time in days
Yeast autolysate and amino acids (GOLBERG and DE MEILLON, 1948b)	85	12·7
Insoluble residue of yeast	95	8·6
Vitamin-free casein (insol.)	60	9·3
17 amino acids (11·64 g/l.)	72	17·3

The synthetic diet therefore involves a slower growth rate, but the survival rate is quite high, and it does allow the role of amino acids and other factors in nutrition to be elucidated.

The indispensability of each amino acid was investigated by omitting each in turn from the basic synthetic diet. The results were quite conclusive (Table 6). There was absolutely no growth when any of the following amino acids were omitted: L-lysine, L-tryptophane, L-histidine, L-phenylalanine, L-leucine, L-*iso*-leucine, L-threonine, L-methionine, L-valine, and L-arginine. Therefore these amino acids are completely essential. In addition, the omission of L-cystine inhibited pupation, although it was not essential for larval development. The omission of L-proline, L-hydroxyproline, or L-serine resulted in a partial arrest of development, principally at the prepupal stage, but also at the first larval stage in the case of L-proline. The omission of L-tyrosine, L-alanine, or glycine had no appreciable effect.

The indispensability of each vitamin was then investigated by omitting each in turn from the basic synthetic diet. The results are shown in Table 7. In the absence of thiamine, nicotinic acid, or calcium pantothenate there was absolutely no growth, and in the absence of riboflavin the larvae did not develop beyond the second instar. Therefore these vitamins are absolutely essential. On the omission of pyridoxine, biotin, or folic acid the larvae grew slowly and failed to pupate. When vitamin B_T or choline chloride was omitted, larval growth and development was poor, although 20 and 35 per cent emergence was obtained. Vitamin B_{12}, *p*-amino-benzoic acid, and *i*-inositol do not appear to be essential. Omission of vitamin B_{12}

lengthened the fourth larval instar; omission of *p*-aminobenzoic acid reduced the emergence by 25 per cent: the omission of *i*-inositol, however, resulted in a slight increase in the survival rate. When glutathione was omitted, some larvae reached the fourth instar but failed to pupate. Since the cystine and glycine components were already present in the synthetic diet, a test was made of the effect of replacing glutathione at 200 mg/l. with glutamic acid at 1·5 g/l. On such a diet the larvae grew slowly, most failing to reach the fourth instar and none pupating.

TABLE 6—EFFECT OF OMITTING INDIVIDUAL AMINO ACID FROM THE CHEMICALLY DEFINED DIET

Amino acid omitted*	No. of larvae		I	II	III	IV	Pupa
L-Cystine	20	No. to instar	20	18	14	0	—
		days in instar	5·0 ± 0·24	3·7 ± 0·31	4·5 ± 0·56	—	—
L-Proline	20	No. to instar	12	12	10	2	2
		days in instar	5·5 ± 0·98	4·0 ± 0·69	5·4 ± 0·88	6·5 ± 0·00	2·0 ± 0·00
L-Hydroxy-proline	20	No. to instar	17	17	17	9	9
		days in instar	6·2 ± 0·63	3·2 ± 0·29	3·0 ± 0·36	5·2 ± 0·08	2·0 ± 0·00
L-Serine	20	No. to instar	19	19	16	10	10
		days in instar	5·9 ± 0·71	3·8 ± 0·41	3·5 ± 0·35	6·4 ± 0·09	2·0 ± 0·00
L-Tyrosine	20	No. to instar	19	18	17	13	13
		days in instar	3·8 ± 0·41	4·3 ± 0·52	4·3 ± 0·38	6·2 ± 0·33	2·0 ± 0·00
Glycine	20	No. to instar	20	19	19	14	13
		days in instar	4·3 ± 0·24	3·3 ± 0·06	4·3 ± 0·29	8·0 ± 0·40	2·0 ± 0·00
L-Alanine	20	No. to instar	20	18	18	16	16
		days in instar	4·1 ± 0·51	3·6 ± 0·07	3·4 ± 0·24	6·5 ± 0·33	2·0 ± 0·00
Full diet	20	No. to instar	20	20	20	15	15
		days in instar	3·4 ± 0·04	3·0 ± 0·06	3·7 ± 0·07	5·8 ± 0·31	2·0 ± 0·00

* When L-leucine, L-*iso*leucine, L-threonine, L-valine, L-phenylalanine, L-lysine, L-arginine, L-histidine, L-tryptophane, or L-methionine was omitted, there was no growth at all.

Finally, the effect on larvae of the omission of glucose, salts, lipids, or RNA from the basic synthetic diet was tested. In the absence of any one of these factors growth was greatly retarded (Table 8), and the omission of the lipid fraction arrested growth at the third instar. When either glucose or inorganic salts were omitted, a few larvae reached the fourth instar but failed to pupate.

On completion of the larval tests, the nutrition of the emerged adults was next investigated. As a control experiment, females were reared on a larval diet of fox chow and yeast and an adult diet of beef blood serum and honey was given on a cotton pad. When dissected 15 days later, 21 out of the 25 females so fed were

found to have their ovaries in Stage V* of development (CHRISTOPHERS, SINTON and COVELL, 1936). When the serum was replaced by 10 essential amino acids

TABLE 7—EFFECT OF OMITTING INDIVIDUAL VITAMIN FROM THE CHEMICALLY DEFINED DIET

Vitamin omitted†	No. of larvae		I	II	III	IV	Pupa
Riboflavin	20	No. to instar	16	0	—	—	—
		days in instar	4·9 ± 0·30	—	—	—	—
Pyridoxine	20	No. to instar	18	18	12	0	—
		days in instar	5·2 ± 0·35	3·5 ± 0·30	4·5 ± 0·34	—	—
Folic acid	20	No. to instar	17	17	13	0	—
		days in instar	6·1 ± 0·41	3·8 ± 0·36	3·3 ± 0·39	—	—
Choline Cl.	20	No. to instar	14	14	13	10	7
		days in instar	4·7 ± 0·40	3·3 ± 0·37	3·0 ± 0·37	5·5 ± 0·29	2·0 ± 0·00
Biotin	20	No. to instar	17	12	3	0	0
		days in instar	4·9 ± 0·39	4·5 ± 0·49	2·8 ± 0·40	—	—
B_T	20	No. to instar	17	16	14	6	4
		days in instar	6·5 ± 0·41	3·2 ± 0·33	2·9 ± 0·23	5·3 ± 0·61	2·0 ± 0·00
Glutathione	20	No. to instar	10	7	5	0	0
		days in instar	10·0 ± 1·28	4·7 ± 0·81	3·5 ± 0·12	—	—
B_{12}	20	No. to instar	19	19	19	14	14
		days in instar	4·0 ± 0·36	3·5 ± 0·22	3·5 ± 0·29	7·6 ± 0·40	2·0 ± 0·00
p-Amino-benzoic acid	20	No. to instar	19	18	14	11	10
		days in instar	4·6 ± 0·29	3·4 ± 0·05	3·3 ± 0·29	5·3 ± 0·44	2·0 ± 0·00
i-Inositol	20	No. to instar	20	20	20	17	17
		days in instar	3·4 ± 0·31	3·9 ± 0·37	3·0 ± 0·41	5·6 ± 0·09	2·0 ± 0·00
Full diet	20	No. to instar	20	20	19	15	15
		days in instar	3·9 ± 0·07	3·3 ± 0·07	3·6 ± 0·27	5·7 ± 0·28	2·0 ± 0·00

† When thiamine, Ca pantothenate, or nicotinic acid was omitted, there was no growth at all.

(Mixture *C*, Table 9) only 18 out of 69 adults so fed reached Stage V of ovarian development. Glucose could be substituted for honey without change in the results.

Females reared on the aseptic synthetic larval diet with sterile precautions were then fed on two amino acid mixtures, *A* and *B* (Table 9). The insects were not dissected, but were kept until they laid eggs. Mixture *B*, which contained 12 amino acids, allowed a higher egg production than Mixture *A*, which contained

* At this stage the eggs are mature and the chorion has developed.

18 amino acids. The eggs so obtained were placed in solution MD No. 185, and they all hatched. Then the effect of omitting individual amino acids from Mixture *B* was investigated; phenylalanine, *iso*leucine, leucine, lysine, threonine, methionine, tryptophane, histidine, valine, and arginine were found to be essential, whereas cystine and glutamic acid could be omitted without inhibiting oviposition.

TABLE 8—EFFECT OF OMITTING GLUCOSE, LIPIDS, RNA, OR INORGANIC SALTS FROM THE CHEMICALLY DEFINED DIET

Compound omitted	No. of larvae		I	II	III	IV	Pupa
Glucose	20	No. to instar	12	12	11	0	—
		days in instar	4·8±0·74	5·3±0·35	5·7±0·42	—	—
Lipids	20	No. to instar	17	9	0	—	—
		days in instar	7·1±0·43	6·2±0·56	—	—	—
RNA	20	No. to instar	18	18	16	0	—
		days in instar	3·7±0·23	5·0±0·23	5·5±0·37	—	—
Inorganic salts	20	No. to instar	14	12	6	0	—
		days in instar	8·8±0·91	4·9±0·41	6·4±0·91	—	—

TABLE 9—ROLE OF AMINO ACIDS IN INDUCING EGG MATURATION AND OVIPOSITION BY ADULTS

Amino acid	Mixture *A* (g/l.)	Mixture *B* (g/l.)	Mixture *C* (g/l.)	No. of eggs/20 females when amino acid omitted from Mixture *B*
DL-Leucine	20·0	7·5	10·0	0
DL-*Iso*leucine	10·0	5·0	10·0	0
DL-Threonine	8·0	3·0	6·0	0
DL-Valine	10·0	10·0	6·0	0
DL-Phenylalanine	7·0	12·0	5·0	0
L-Lysine	9·0	7·5	9·0	0
L-Arginine	5·0	3·8	6·0	0
L-Histidine	7·0	1·5	2·5	0
L-Tryptophane	4·0	3·0	2·0	0
L-Methionine	2·0	1·5	2·0	0
L-Cystine	2·0	1·5	—	175
L-Proline	5·0	—	—	—
DL-Serine	2·0	—	—	—
Glycine	5·0	—	—	—
L-Alanine	6·0	—	—	—
L-Tyrosine	4·0	—	—	—
L-Aspartic acid	10·0	—	—	—
L-Glutamic acid	10·0	10·0	—	97
Oviposition of 20 females on Mixture *B*				180
Oviposition of 20 females on Mixture *A*				95

DISCUSSION

The investigations here reported have been concerned with the elimination from the diet of any yeast material not chemically defined, and finally the replacement of protein by individual amino acids. GOLBERG and DE MEILLON (1948b) obtained development on diets containing amino acids, but only if they added twice as much nitrogenous material in the form of yeast autolysate. This was prepared by suspending dried yeast powder in water at 50°C for 24 hr, or was purchased as Bacto Yeast Extract. It is possible that the yeast autolysate they employed at that time contained small amounts of certain vitamins, since we were unable to obtain growth with this commercial yeast autolysate in 1955. In the earlier work, GOLBERG, DE MEILLON and LAVOIPIERRE (1945) had obtained development when the dietary protein was supplied by the insoluble residue of yeast; the present experiments indicate that this latter dietary material also supplied lipids, RNA, traces of glutathione, biotin, and possibly vitamins B_{12} and B_T. Thus it becomes possible to replace the insoluble residue of yeast with casein if it is supplemented by lipids, RNA, glutathione, and the three vitamins mentioned.

The advantage of insoluble protein over dissolved protein, observed at this stage, made it clear that if protein was to be replaced by amino acids, the amount of molecules in solution must be restricted. Previous workers had not encountered this problem, since they supplied the nitrogenous nutrition in the form of protein; and even GOLBERG and DE MEILLON (1948b), in investigating the role of individual amino acids, supplied the greater part of the nitrogen as the protein in yeast autolysate. However, in a communication published 6 months after the present experiments had been completed, LEA, DIMOND and DELONG (1956b) reported that they had been able to substitute amino acids for casein by incorporating them as a solid fraction in agar. In the present experiments the larvae can survive and utilize dissolved amino acids, provided the osmotic pressure of the diet does not exceed $\Delta 0{\cdot}40$°C.

The osmotic pressure of the haemolymph of *A. aegypti* larvae has been reported by WIGGLESWORTH (1938) to be equivalent to 0·75–0·89 per cent NaCl; whereas they can maintain a constant osmotic pressure of the haemolymph in ambient solutions equivalent to 0·75 per cent NaCl or less, they are unable to do so in hypertonic solution. The same increase in haemolymph osmotic pressure in hypertonic solution is also shown by *Culex pipiens*. On the other hand, the larvae of *Aedes detritus* maintain a moderate haemolymph osmotic pressure even when the ambient concentration reaches 6·0 per cent NaCl, the haemolymph concentration not exceeding 1·3 per cent NaCl, and even in 2·0 per cent NaCl the haemolymph concentration is still below 1·0 per cent NaCl (BEADLE, 1939). *Culex fatigans* has similar abilities (WOODHILL, 1938). It is evident that the species of the former class, such as *A. aegypti*, cannot take in food through their midgut in hypertonic solutions without raising the haemolymph osmotic pressure; since they are unable to do this, they do not grow but die at the higher osmotic pressures.

The present experiments show that the amino acids which are absolutely essential, in that the omission of any one inhibits development, are L-*iso*leucine,

L-leucine, L-lysine, L-methionine, L-arginine, L-histidine, L-phenylalanine, L-threonine, L-tryptophane, and L-valine. These 10 amino acids, which are listed as the 10 essential compounds in mammalian nutrition, have also been shown to be essential for *Attagenus* sp. (MOORE, 1946), *Tribolium confusum* (LEMONDE and BERNARD, 1951; FRAENKEL and PRINTY, 1954), and *Hylemya antiqua* (FRIEND, BACKS and CASS, 1956). These are 10 of the essential amino acids for *Pseudosarcophaga affinis* larvae, but glycine constitutes the eleventh (HOUSE, 1954c). These are also the 10 found essential for *A. aegypti* larvae by GOLBERG and DE MEILLON (1948b), who also reported that glycine is essential and that tyrosine could replace phenylalanine. These 10 were also essential for *Drosophila melanogaster* (LAFON, 1939; RUDKIN and SCHULTZ, 1947; HINTON, NOYES and ELLIS, 1951), with serine constituting the eleventh (HINTON, NOYES and ELLIS, 1951). In the nutrition of *Blattella germanica* threonine and phenylalanine are not essential, whereas alanine constitutes the ninth essential amino acid (HOUSE, 1949; NOLAND and BAUMAN, 1951; HILCHEY, 1953).

In the present experiments the omission of L-cystine completely inhibited pupation, and even the presence of glutathione failed to assist the formation of pupae. Lack of cystine was found to inhibit ecdysis or pupation in *B. germanica* (HOUSE, 1949b) and *Lucilia sericata* (MICHELBACHER, HOSKINS and HERMS, 1932). GOLBERG and DE MEILLON (1948b) reported that the omission of L-cystine from the diet of *A. aegypti* inhibited emergence in the majority of cases.

The present experiments showed that the omission of proline, hydroxyproline, or serine resulted in arrest of development at the first and fourth larval instars. Proline has been found by HILCHEY (1953) to be required by males of *B. germanica*. Surprisingly enough, the omission of hydroxyproline from the diet allowed larvae of *H. antiqua* to develop at a slightly faster rate (FRIEND, BACKS and CASS, 1956). Serine has been reported by TRAGER (1947) as improving growth when added to the 10 essential amino acids. It is required for the normal growth of male *B. germanica* (HILCHEY, 1953) and *D. melanogaster* (HINTON, NOYES and ELLIS, 1951); and its absence retards the larval growth rate of *P. affinis* (HOUSE, 1954c). Yet FRIEND, BACKS and CASS (1956) found that its omission significantly improved the growth rate of *H. antiqua*, whereas HINTON, NOYES and ELLIS (1951) noted that D-serine was extremely toxic and L-serine slightly toxic to the larvae of *D. melanogaster*.

The omission of glycine, tyrosine, or alanine from the diet did not affect the larval growth of *A. aegypti*. Yet glycine was reported to be essential for *A. aegypti* by GOLBERG and DE MEILLON (1948b); and it was found to be indispensable for *P. affinis* by HOUSE (1954c) and for *Oryzaephilis surinamensis* by DAVIS (1956). Although glutathione was added in the present experiments its amount was small, and it would appear that *A. aegypti* resembles most animals in synthesizing the compound. Tyrosine has been found to increase the growth rate of *P. affinis* slightly (HOUSE, 1954c). Alanine has been shown to be essential for *B. germanica* (HILCHEY, 1953) and to stimulate the growth of *P. affinis* (HOUSE, 1954c), but DL-alanine has been found by DAVIS (1956) to be toxic for *O. surinamensis*.

The experiments here reported show clearly that thiamine, riboflavine, pantothenic acid, and nicotinic acid are essential in the nutrition of *A. aegypti*, in that there is no larval growth in their absence. The indispensability of these four vitamins has been proved also in the following 19 insects: *Blattella*, *Attagenus*, *Lasioderma*, *Tribolium* (*confusum* and *castaneum*), *Dermestes*, *Oryzaephilus*, *Palorus*, *Ptinus*, *Tenebrio*, *Plodia*, *Ephestia* (*kuehniella* and *elutella*), *Tineola*, *Culex*, *Lucilia*, *Drosophila*, *Hylemya*, and *Pseudosarcophaga* (ALBRITTON, 1954).

Five other vitamins were found to be essential in that growth was incomplete when they were omitted from the diet. In the absence of pyridoxine, biotin, or folic acid the larvae failed to pupate. In the absence of vitamin B_T or choline chloride only a small percentage reached the adult stage.

In the absence of pyridoxine, 60 per cent of the larvae reached the fourth instar, but none succeeded in pupating. It has been the general experience that this vitamin is indispensable in insect nutrition, although pyridoxine is not essential for *P. affinis* (HOUSE, 1954b).

In the absence of biotin, only 15 per cent of the larvae reached the fourth instar. It is probable that this amount of growth was induced by the lecithin in the diet, since TRAGER (1948) has shown that lecithin can partially replace biotin for *A. aegypti* and allows the growth of larvae but not their metamorphosis. In all previous studies of other species biotin has been shown to be essential.

In the absence of folic acid, 65 per cent of the larvae reached the fourth instar, but none pupated. It has been the general experience that this vitamin is essential, and that its absence inhibits pupation of the following dipterous species: *D. melanogaster* (BEGG and ROBERTSON, 1950), *H. antiqua* (FRIEND, BACKS and CASS, 1956), *C. molestus* (LICHTENSTEIN, 1948), and *A. aegypti* (GOLBERG, DE MEILLON and LAVOIPIERRE, 1945). On the other hand, omission of folic acid did not prevent the pupation of *P. affinis* (HOUSE, 1954b), and actually stimulated the growth of *B. germanica* (NOLAND, LILLY and BAUMANN, 1949).

In the absence of vitamin B_T, the growth rate was greatly retarded in the early instars, and only 20 per cent reached the adult stage. This nitrogenous base (carnitine) has hitherto been shown to be essential for *Tenebrio molitor* (FRAENKEL, BLEWETT and COLES, 1950), *Palorus ratzeburgi* (COOPER and FRAENKEL, 1952), and *Tribolium confusum* (FRENCH and FRAENKEL, 1954). HINTON, NOYES and ELLIS (1951) found that its absence had no effect on *D. melanogaster*.

In the absence of choline chloride, only 35 per cent reached the adult stage. This nitrogenous base can be synthesized by vertebrates from the amino acids serine and methionine, and it is possible that this conversion may take place in insects. Nevertheless, the presence of choline is usually a requisite in the insect diet, although normal growth of *O. surinamensis* (FRAENKEL and BLEWETT, 1943b, c) and *Sitodrepa paniceum* (PANT and FRAENKEL, 1950) has been obtained in its absence.

These experiments shed some light on the nutritional importance of vitamin B_{12}, *i*-inositol, and *p*-aminobenzoic acid. The absence of vitamin B_{12} did not prevent complete development of *A. aegypti*, as has been the general experience with other insects. However, in its absence the larvae took a longer time to pass

from the fourth instar to the pupal stage. The addition of vitamin B_{12} to the diet has been found to increase the percentage pupation of *P. affinis* (HOUSE, 1954b) and *D. melanogaster* (HINTON, NOYES and ELLIS, 1951). As in other insects, *i*-inositol is not essential in the nutrition of *A. aegypti*, its absence causing no decrease in growth. The omission of *p*-aminobenzoic acid did not decrease the growth rate of those *A. aegypti* which complete their life cycle, thus confirming the experience with insects in general. However, it did decrease the number of individuals passing from one instar to the next, and in this connexion one should mention the observations of FRAENKEL and BLEWETT (1943d) that the average growth rate of *Ptinus tectus* was decreased in the absence of *p*-aminobenzoic acid.

Glutathione proved to be a very important requirement in the nutrition of *A. aegypti*; only 25 per cent of the larvae reached the fourth larval instar in 15·4 days. An attempt to replace this tripeptide with glutamic acid in the diet proved unsuccessful. The larvae of *A. aegypti* are therefore the only insects which have so far been shown to require glutathione. TRAGER (1948) had found that addition of glutathione to a casein diet increased the growth rate of *A. aegypti*, but GOLBERG, DE MEILLON and LAVOIPIERRE (1945) found that it did not enhance a diet based on the insoluble residue of yeast. No other workers have investigated the effects of this compound with the exception of HOUSE (1954d), who reported that the addition of glutathione to a synthetic diet based on amino acids increased the percentage pupation of *P. affinis*.

In addition to the vitamin and amino acid requirements, the remaining dietary elements—lipids, inorganic salts, carbohydrate, and RNA—cannot be omitted without seriously impairing the growth of *A. aegypti*. In the absence of lipids, the development never passed the third instar. In these experiments the lipids involved a 1 : 2 : 1 mixture of cholesterol, lecithin, and cephalin, the minimal lipid diet of GOLBERG and DE MEILLON (1948a), although TRAGER (1948) has shown that cholesterol is the only essential lipid.

In the absence of inorganic salts, 30 per cent of the larvae reached the fourth instar. The salt mixture employed, which was MD No. 185 mixture first used for insects by FRAENKEL and BLEWETT (1943a), contains only 8 inorganic elements (K, Na, Mg, Ca, Cl, S, P, Fe), but was still sufficient for the normal growth of *A. aegypti*, as for other insects. The mixture is high in calcium and phosphate. The indispensability of calcium in the nutrition of mosquito larvae has been emphasized by TRAGER (1936), FROST, HERMS and HOSKINS (1936), and BATES (1939).

When carbohydrate was omitted from the diet, 55 per cent of the larvae reached the fourth instar but none pupated. The artificial diet used for mosquito larvae by TRAGER (1948) included 12 per cent sucrose. GOLBERG and DE MEILLON (1948b) used 20 per cent of glucose, and this proportion was used in the present experiments. It is evident that *A. aegypti* larvae do not resemble *Phormia regina* (BUST and FRAENKEL, 1955) and other muscoid larvae in being able to dispense with carbohydrate: it would appear rather that they are in a class with *D. melanogaster*, *P. affinis*, and *H. antiqua* larvae, for which soluble sugars in the proportions of 18 to

40 per cent have been employed (HINTON, NOYES and ELLIS, 1951; HOUSE, 1954a, d; FRIEND, BACKS and CASS, 1956).

In the absence of RNA, nearly all the larvae reached the fourth instar, but growth was retarded and pupation was inhibited. SUBBAROW and TRAGER (1940) had observed that the addition of RNA would increase the growth rate of *A. aegypti* on a diet based on liver extract and heat-killed yeast. The absence of RNA in the diet of *P. affinis* (HOUSE, 1954d) decreased the growth rate and the number of larvae reaching the pupal stage. HINTON, NOYES and ELLIS (1951) found that the toxicity of serine to *D. melanogaster* was reduced by RNA.

Using the complete synthetic diet developed along the lines indicated, it is now possible to obtain oviposition of viable eggs from female mosquitoes thus reared aseptically by feeding them a mixture of amino acids after emergence. The results on adult nutrition are in general agreement with those of DIMOND *et al.* (1956), who reared the larvae on an undefined stock diet which was not aseptic. On a mixture of 12 amino acids (their Medium *D*) these workers obtained an average egg production of 40 per female in 14 days; on this same mixture (our Mixture *B*) fed to aseptic adults, a production of 9 eggs per female in 6 days was obtained. On omitting individual amino acids it was found in our experiments that the following 10 amino acids were completely essential: arginine, histidine, *iso*leucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine. These were the 10 amino acids that DIMOND *et al.* (1956) found to be essential in tests removing each one from a diet of 13 amino acids (their Medium *A*). Whereas glutamic acid was found to be unessential in both investigations, there is a lack of agreement on the role of cystine. DIMOND *et al.* (1956) found that the omission of cystine decreased the egg production, whereas in the present experiment the omission of this amino acid was without effect.

It is interesting to find that the adult female *A. aegypti* require, in order to mature their eggs, exactly the same 10 amino acids as the larvae require for growth and development. In addition, the larvae need cystine for pupation, and their growth rate is stimulated by serine, proline, and hydroxyproline; egg production in the adult is also stimulated by glutamic acid and cystine (DIMOND *et al.*, 1956). Glycine, tyrosine, and alanine are not required by either the larvae or the adult.

SUMMARY

The nutritional requirements of the yellow-fever mosquito *Aedes aegypti* L. have been investigated throughout the complete life cycle. Aseptic precautions were taken throughout the larval stage and until emergence. The adults were fed on a complete synthetic diet. It has been found that the larvae grew on a complete synthetic diet consisting of 17 amino acids, 12 vitamins, glutathione, 8 inorganic elements, 3 lipids, RNA, and glucose, provided the osmotic pressure of the diet did not exceed Δ0·4°C. The adults so obtained laid viable eggs when they were fed a mixture of 12 amino acids with glucose or fructose. The following amino acids were found to be essential for both larval and adult nutrition: arginine, *iso*leucine, leucine, lysine, phenylalanine, methionine, threonine, tryptophane,

valine, and histidine; these are the 10 indispensable amino acids of mammalian nutrition. The following 9 vitamins are essential for larval nutrition: thiamine, riboflavin, pantothenic acid, nicotinic acid, pyridoxine, biotin, folic acid, vitamin B_T, and choline chloride. Vitamins are not required in adult nutrition. Glutathione, glucose, inorganic salts, lipids, and RNA are also required for complete growth and development of the larvae.

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A STUDY OF NITROGEN AND URIC ACID PATTERNS IN THE EXCRETA AND BODY TISSUES OF ADULT *AEDES AEGYPTI*

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Abstract—The studies reported in this paper have been undertaken to establish eventually, if possible, metabolic changes in the physiological pattern of the mosquito *Aedes aegypti*, which may be related to demonstrated changes in its susceptibility or resistance to infection produced by various biological, biochemical, or physical factors. Preliminary studies of nitrogen metabolism in this animal have shown that immediately after emergence as adults, *A. aegypti* excrete considerable amounts of nitrogenous material, the bulk of which has been identified by chemical, physical, and enzymatic methods as uric acid. Since the output of uric acid decreases rapidly thereafter, and remains at extremely low levels during the remainder of the adult existence of the mosquitoes, so long as they are maintained on sucrose alone, these data suggest that immediately following emergence mosquitoes are excreting essentially the products of pupal metamorphosis rather than the products of adult metabolism. The data also indicate that ingested proteins are utilized by the mosquito, only if they are converted to and excreted as uric acid, since there is an immediate and extreme rise in the proportional output of uric acid following a blood meal, whereas in mosquitoes maintained on plasma or lysed red cells only a very small proportion of the excreted nitrogen is uric acid. This assumption is supported by the comparative tissue nitrogen levels of mosquitoes maintained on these diets. Finally, the data suggest that all the proteins necessary for adult existence are obtained by the female adult mosquito during its larval developmental period, and in the absence of any egg-laying activity it is capable of conserving its nitrogen resources and maintaining itself adequately at a minimal level of protein katabolism.

INTRODUCTION

IT HAS been shown that certain biochemical and physical factors markedly influence the innate immunity of the mosquito *Aedes aegypti* to infection with the malarial parasite *Plasmodium gallinaceum* (TERZIAN *et al.*, 1953; TERZIAN, 1953). The studies to be reported in this series have been undertaken to elucidate some of the biochemical characteristics of the mosquito under normal and experimental conditions in an effort to relate, eventually, possible changes in these characteristics to changes in their susceptibility or resistance to infection. The present report deals with the isolation and characterization of uric acid from mosquito excreta and with analyses of the nitrogenous constituents of mosquito excreta, as well as analyses of the tissue nitrogen levels of normal mosquitoes and mosquitoes maintained on different dietary supplements.

MATERIALS AND METHODS

A laboratory strain of *A. aegypti* mosquitoes maintained at a constant temperature of 26·5°C and a relative humidity of 75 per cent was used for these experiments. The larvae were grown in glass crocks and fed solely on guinea-pig food pellets added in appropriate amounts from time to time during the larval development period. The adult mosquitoes were set up in lucite cylinders in groups of 80–100 females, and for each experiment mosquitoes derived from the same larval colony were used. The adult female mosquitoes were routinely fed on 4 per cent sucrose solutions made available 24 hr after pupal emergence was completed, and replaced daily during the course of an experiment. Dietary supplements, such as plasma or washed red cells, whenever required, were added in appropriate concentration to the regular 4 per cent sucrose solutions. Whole blood was administered to mosquitoes by permitting them to engorge on young chicks.

The excreta of the mosquitoes was collected on Whatman No. 1 filter paper placed on the bottom of the cylinders over Teflon sheets. The collection of excreta was begun as soon as the pupae began to emerge as adults, and continued during the course of an experiment in the following manner. Filter papers were placed in the cylinders immediately after the pupae were harvested and the excreta of all the emerging adults was collected before they were placed on the sugar solutions. This collection was designated as sample A. As soon as emergence was completed (48–72 hr) this paper was removed and replaced with new filter paper, and the excreta collected for the first period of 7 days was designated as sample B. At this time the paper was again replaced and the excreta collected during the second 7 day period was labelled sample C. Samples were collected weekly thereafter and labelled according to the convention noted above until the experiment was terminated. Each series represented the collection from approximately 2000 female mosquitoes.

Prior to extraction, filter papers containing the mosquito excreta were examined under u.v. light for the presence of contaminating substances such as spilled food solutions, which, if present, were cut out of the papers. The excreta was then extracted by adding distilled water to the papers and macerating them in a Waring blender at 10°C. The resulting suspension was filtered and washed with distilled water and the filtrate then lyophilized. The solid material thus obtained was used for all subsequent determinations and analyses. For the quantitative analyses of the excreta, total nitrogen was determined by the Kjeldahl method, and uric acid by the method of KALCKAR (1947) as modified by PRAETORIUS (1949). The uricase required for the latter method was prepared from fresh pig liver according to the procedure of MÜLLER and BAUER (1953).

For weight and tissue nitrogen determinations, mosquitoes were set up in cylinders in groups similar to those used for the collection of excreta and withdrawn for these determinations on the same schedule. For dry weight and tissue nitrogen determinations, mosquitoes were dried in a vacuum desiccator over P_2O_5 until weight was constant.

RESULTS

Isolation of uric acid

Although insects in general excrete nitrogenous wastes principally as uric acid, this compound has not been isolated or identified by physical or chemical methods in the excreta of adult mosquitoes. To isolate uric acid from this source, pooled samples of the lyophilized excreta were suspended in minimal amounts of distilled water, and then either N Na_2CO_3 or NaOH was added drop by drop until most of the particles were dissolved. Following clarification with activated charcoal, the filtrate was acidified with N HCl, using Congo red as an indicator. The crystals which precipitated from the solution following cooling in an ice bath were filtered and washed with distilled water, then redissolved in alkali and reprecipitated with acid. The process of solution and precipitation was repeated twice more. The resulting crystals obtained by this procedure are thin plates which resemble exactly a doubly recrystallized commercial preparation of uric acid (Found : C, 35·92 ; H, 2·27. Calc. for $C_5H_4N_4O_3$: C, 35·72 ; H, 2·40 per cent).

The crystals give a murexide test and a blue colour with phospho-tungstic acid. Equimolar solutions of the uric acid from mosquito excreta and that of a known sample are both destroyed by uricase giving identical decay curves, and both give the same absorption spectrum in a Carey recording spectrophotometer. Both showed the same minimum and maximum peaks: minimum, 220 mμ and 260 mμ; maximum, 291–293 mμ and 235 mμ. With the ascending technique of paper chromatography and *tert.*-butanol–formic acid–H_2O (70 : 15 : 15) the *Rf* values for both the unknown and standard uric acid were 0·17.

Nitrogen excretion of the adult mosquito

Fig. 1 presents the initial and weekly total nitrogen and uric acid nitrogen levels in the excreta of *A. aegypti* maintained on 4 per cent sucrose. The non-uric acid nitrogen was obtained by difference between these two determinations. It may be seen from these data that during the first 24–72 hr following emergence as adults (sample A) there is a maximal output of nitrogenous material in the mosquito excreta, the bulk of which is uric acid. During the first 7 days following emergence (sample B) there is still a very considerable amount of nitrogen excreted, but the amount of uric acid is substantially reduced and constitutes a significantly lower proportion of the total nitrogen. Thus, whereas in sample A, 82 per cent of the total nitrogen was uric acid, in sample B only 31 per cent was uric acid. During the next 7 day period (sample C) the nitrogen excreted decreases again very considerably while the proportion of uric acid is still further reduced to 15 per cent of the total nitrogen. During the third week, however, the amount of total nitrogen in the excreta begins to rise (sample D), and in the fourth week (sample E) the rise in the amount of nitrogen excreted is even more pronounced and significant. At the same time, the amount of uric acid excreted continues to decline until in the fourth week (sample E) it is less than 4 per cent of the total nitrogen excreted. At the end of the fourth week the mosquitoes were allowed to feed on normal chicks, and in the excreta collected following the blood meal (sample Eb) there was an

immediate rise in the amount of nitrogen excreted, in which, however, as in sample A, the amount of uric acid rose to 79 per cent of the total nitrogen. It may be noted here that the values for uric acid in the various excreta samples following a blood meal have been corrected for the uric acid content of the chick blood

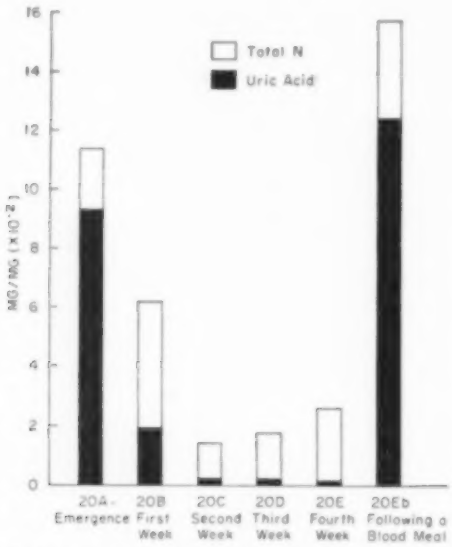

FIG. 1. The total nitrogen and uric acid content of the excreta of adult *Aedes aegypti* maintained on sugar, collected on emergence and at weekly intervals, and following a blood meal.

ingested, and the correction has been calculated to be only 0·0002 mg uric acid N/mg.

Fig. 2 presents the data from another comparable experiment in which the mosquitoes were again maintained on 4 per cent sucrose solutions but in which they received, instead, a blood meal at the end of the first week. Again there was a high initial output of nitrogen with a proportionately large amount of uric acid (sample A) and similarly, during the first 7 days following emergence, the nitrogen output decreased considerably while the proportion of uric acid was again reduced to 30 per cent of the total nitrogen excreted (sample B). As in the first experiment cited, however, following the blood meal the mosquitoes excreted a large amount of nitrogen containing a high proportion of uric acid (sample C). And once again, during the fourth week (sample E) there was an increase in the amount of nitrogen excreted as compared to the output during the third week (sample D). Meanwhile, in samples D and E, the uric acid output returned again to comparatively constant and low levels.

In view of the fact that whole blood ingested naturally by the mosquito is largely converted to uric acid, it is of interest to note that mosquitoes maintained

on chick plasma solutions, or solutions of lysed chick red cells, failed to synthesize uric acid to the same extent from the separated blood elements as from whole blood taken in by feeding on a host animal. Thus, as shown in Fig. 3, the uric acid produced by mosquitoes maintained on plasma (37D) amounted to only

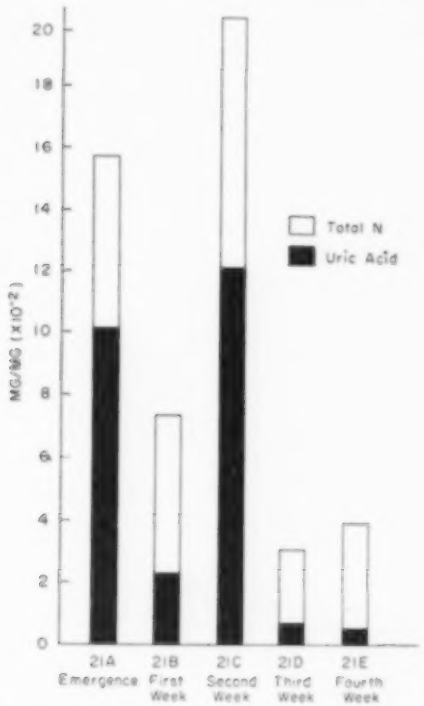

FIG. 2. The total nitrogen and uric acid content of the excreta of adult *Aedes aegypti* maintained on sugar, but allowed a single blood meal during the second week following emergence.

9·1 per cent of the total nitrogen, while the uric acid of mosquitoes maintained on lysed red cells (38C) was 18·2 per cent of the total nitrogen excreted. However, in another group of mosquitoes which had taken up whole blood naturally (35Fb), the uric acid content rose to 54 per cent of the total nitrogen excreted. It would appear, therefore, as if certain essential factors or co-factors, required for the synthesis of uric acid from whole blood, are either removed or changed when blood is fractionated into plasma and red cells. The probability that there may be some significant biochemical change in blood when it is fractionated is further borne out by studies in which it has been shown that ageing mosquitoes which have become resistant to infection, when allowed to take a blood meal, become again as susceptible to infection as young mosquitoes, whereas ageing mosquitoes which have been fed on plasma, lysed red cells, or plasma and lysed red cells combined become

instead even more resistant to infection than they normally would have become as a result of ageing (TERZIAN *et al.*, 1956).

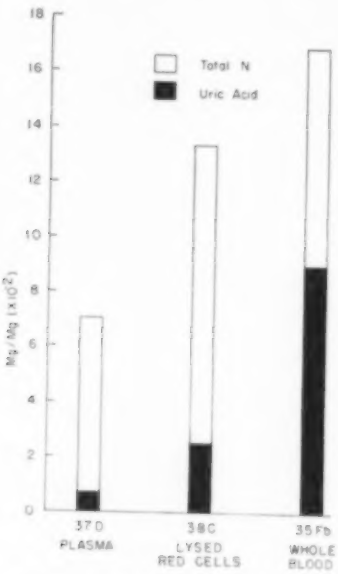

FIG. 3. The comparative biosynthesis of uric acid as shown in the excreta of *Aedes aegypti* adults fed on plasma, lysed red cells and whole blood.

Tissue nitrogen levels of adult mosquitoes

In consequence of the continuing nitrogen depletion evidenced by the analyses of the excreta of mosquitoes fed on sucrose, it was necessary to determine the levels of tissue nitrogen in mosquitoes similarly maintained on sucrose over the same periods of time. Table 1 shows, first, that the wet weights and the dry weights parallel each other, and that the moisture content of the mosquitoes remains comparatively constant over the 5 week period, and second, that there is an increase in the weight of the animal during the first week following emergence, which continues during the second week when it reaches its maximum value. Thereafter, however, the weight begins to decline and continues to decrease during the remaining 3 weeks of the experiment. On the other hand, the percentage of tissue nitrogen declines during the first 2 weeks but then begins to increase, continues to increase during the next 3 weeks, and reaches its maximum at the end of this period. A comparison of the data for the dry weights and the percentages of tissue nitrogen of these mosquitoes over the 5 week period indicates a relation in which the values for the one set of data appear to be the reciprocals of the other. It appears not unreasonable to assume, therefore, that the nitrogen content of these animals remains comparatively constant during this period of time. Thus, even though there is continuing protein metabolism as

analyses of the excreta show, nevertheless, these data indicate that this metabolism is maintained at a level which effectively conserves the nitrogen resources of the adult in the absence of any protein intake.

TABLE 1—THE PERCENTAGE NITROGEN AND WET AND DRY WEIGHT OF ADULT FEMALE *Aedes aegypti* MOSQUITOES

Sample	Mosquito age (days)	Per cent nitrogen	Wet wt/mosq. (mg)	Dry wt/mosq. (mg)	Per cent moisture
A	1	8·22	2·42	0·84	65·15
B	7	7·52	2·45	0·90	63·23
C	14	6·58	2·58	0·94	63·43
D	21	7·27	2·23	0·81	63·60
E	28	7·76	2·25	0·79	64·74
F	35	8·52	2·04	0·69	66·31

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In the experiment shown in Table 2, in addition to a series of mosquitoes maintained on 4 per cent sucrose, another series was maintained on 10 per cent

TABLE 2—THE PERCENTAGE NITROGEN AND WET AND DRY WEIGHTS OF ADULT FEMALE *Aedes aegypti* MOSQUITOES

Sample	Mosquito age (days)	Per cent nitrogen	Wet wt/mosq. (mg)	Dry wt/mosq. (mg)
	(Maintained on 4% sucrose)			
A	1	9·5	2·27	0·72
B	7	7·8	2·61	0·87
C	14	8·3	2·29	0·74
D	21	9·0	2·16	0·67
E	28	9·6	2·24	0·59
F	35	9·7	2·26	0·63
	(Maintained on 4% sucrose containing 10% plasma)			
B	7	8·1	2·42	0·80
C	14	7·4	2·66	0·91
D	21	7·9	2·57	0·92
E	28	9·4	2·29	0·71
F	35	9·6	2·30	0·73
	(Maintained on 4% sucrose and blood meals)			
B	7	8·7		0·84
C	14	9·2		0·82
D	21	9·3		0·70
E	28	9·0		0·79
F	35	8·9		0·71

plasma, that is, 10 ml of chick plasma added to 90 ml of 4 per cent sucrose. The third series was given a blood meal on emergence, and the first group was removed for analysis on the seventh day following the blood meal (sample B). At that time

the remaining mosquitoes were again allowed to feed on a normal chick, and 7 days after this blood meal another group was then removed for nitrogen determinations (sample C). The same procedure was followed for the remaining groups in the series, so that finally the mosquitoes removed for sample F had received five blood meals by the time the experiment was terminated.

In this experiment, as before, mosquitoes maintained on sucrose alone showed the same decrease in percentage of tissue nitrogen during the week after emergence but again, during the following weeks, there was a gradual increase in tissue nitrogen so that by the end of the fifth week the levels of tissue nitrogen of the ageing mosquitoes equalled those of the young, newly emerged individuals. In the mosquitoes maintained on plasma the percentage of tissue nitrogen levels remained lower during the first 3 weeks after emergence but during the fourth and fifth weeks the levels returned again to those of newly emerged adults, thus resembling to a considerable extent the tissue nitrogen patterns of the mosquitoes maintained on sugar alone, and indicating that little or none of the plasma was utilized to add to their body stores of protein or nitrogen. On the other hand, in spite of continuing egg production the tissue nitrogen levels of the mosquitoes which received blood meals during each week remained comparatively constant and there was little fluctuation during the 5 week period, indicating that these mosquitoes utilized the ingested blood to compensate for the body losses of nitrogen inevitably resulting from the production of large numbers of eggs. Since it has already been shown that mosquitoes maintained on plasma excreted very little uric acid as compared to the uric acid output of mosquitoes allowed to take blood meals, these data would appear to suggest, therefore, that only those proteins which are largely converted to and excreted as uric acid are assimilated or utilized by the mosquito.

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THE PRODUCTION OF SEX ATTRACTANT SUBSTANCE AND OF OÖTHECAE BY THE NORMAL AND IRRADIATED AMERICAN COCKROACH, *PERIPLANETA AMERICANA* L.

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Abstract—A correspondence has been shown to exist between the production of an attractant substance and the mating period of *Periplaneta americana*. The attractant is produced principally by virgin females and, sporadically, by mated females, and is conducive to mating; however, mating depresses the production of attractant. Manipulation of the female can depress the production of attractant. Production of attractant normally decreases with age and as oöthecal production increases. Mating induces an increase in oöthecal production, especially among younger cockroaches. Cathode-ray irradiation damages oöthecal and attractant production. With oöthecal production totally inhibited by irradiation, the females recovered their capacity to produce attractant and exceeded the normal yield. A relationship is indicated between attractant production and the processes which regulate ovulation.

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INTRODUCTION

THE sex attractant produced by the female American cockroach has been studied from three aspects: (1) its production under different conditions; (2) an assay for the attractant as measured by the response of male cockroaches (WHARTON *et al.*, 1954a, b); and (3) its extraction, concentration, and purification. This paper deals with the first of these.

ROTH and WILLIS (1952) found that paper taken from jars containing virgin females of *Periplaneta americana* stimulated males to overt sexual activity, whereas paper taken from jars containing old, non-virgin females failed to do so, indicating that the sex attractant is produced chiefly by virgin females. These authors noted that the approach of the American cockroach to the mating act is direct, lacking in courtship, but accompanied by characteristic motions. We have confirmed these observations, and find further that the effect of the receptive female or of paper impregnated with the attractant is to arouse the interest of the male, as manifested first by its sudden alertness, then movement of the antennae, then active search for the source of odour, then more or less vigorous fluttering of the wings, usually accompanied by protrusion of the abdomen. Repeated tests with our extracts have consistently shown that this sequence of activity corresponds with the influence of increasing concentrations of attractant (WHARTON *et al.*, 1954a). Except for copulation, wing flutter (when it occurs in the presence of a sex stimulus) is the surest visible sexual response and is a certain indication of the

recognition of the specific odour by the male. It is on this response that we have based our assay of the attractant (WHARTON *et al.*, 1954b).

EXPERIMENTAL

Production of attractant

The attractant is obtainable by extraction from the virgin female cockroach or from objects with which it has been in contact during the period of emission. It occurs in minute quantities; nevertheless, the attractant from a highly productive virgin female kept on filter paper in a 250 ml beaker may so permeate the paper in a period of 2 hr that this paper, when placed in a jar with a dozen males, may stimulate several of them sexually.

To test for the production of attractant, we keep isolated females in 250 ml beakers lined at the bottom with a disk of filter paper which is removed every week for testing and replaced with a new paper. We maintain several groups of male cockroaches arranged in separate jars containing 11 insects each, and the paper from each female is assigned to its own group of 66 males throughout the experiment. The number of males responding to the weekly accumulation of attractant on the paper is the basis of comparison in this simple type of test. Over a period of weeks a reliable set of responses may be obtained and compared with controls that are run simultaneously.

Both male and female nymphs of *Blattella germanica* can stimulate adult males sexually (ROTH and WILLIS, 1952). We have found with *P. americana*, however, that nymphs ordinarily do not produce attractant, at least in detectable quantity. Nevertheless, one of the most productive of our cockroaches was a nymph which stayed in her last instar for 5 months. During the last 2 months prior to emergence she produced attractant consistently. Attractance could not be demonstrated on the second or third day after she became an adult, but had definitely returned at the end of one week. This adult cockroach was presented to males on several occasions but failed to mate.

In general, the newly emerged female produces very little attractant. After a week, however, the paper, and the cockroach itself, may be quite active in stimulating males; maximum production of attractant is usually attained by the second week. The level of attractant production subsides gradually over a period of several months, as we have observed with many females (Figs. 1 and 4). Fig. 1 covers the first 8 weeks and contrasts the slow decline in attractant production in unmated cockroaches with the abrupt decline that occurs when the insects are bred. The solid lines are lines of regression which represent the trend of response by males to normal (i.e. not bred) and bred groups of females, respectively, as defined by the method of least squares. The line of regression for the bred group was calculated from the first week after breeding. The broken line in the figure represents the actual response of the males to attractant from females which have been bred. It shows the drastic drop that occurs in production of attractant after breeding. The drop occurs within a period of 18 hr after mating, as actually demonstrated both by presenting the papers and the mated females to males and

noting the relative lack of interest on the part of the males. To determine the effect of mating we exposed each of twenty-five females to eleven males in combined day and electric light and observed the insects in copula. After copulation the

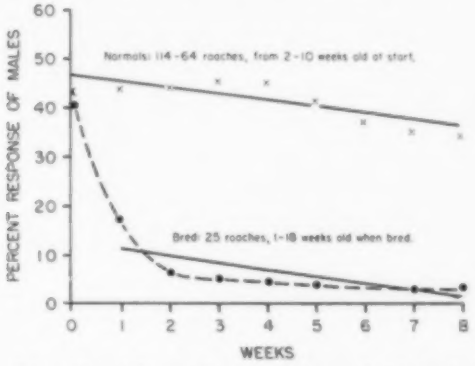

FIG. 1. Effect of breeding (observed) on weekly production of attractant by female *P. americana*. Solid lines show trend of response; the line for the bred group was calculated from the first week after breeding. Broken line shows actual curve of data representing responses of males to bred females, except that response at week zero was obtained before breeding. Note sharp drop in response to attractant from females 1 week after breeding. Test begun with 114 females, reduced to 64 by withdrawal for breeding.

female was returned to its freshly lined container and tested weekly as before. It was found, as shown in Fig. 1, that the production of attractant was permanently reduced by mating. (The terms mating and breeding are used interchangeably to mean that the female has been in copula with the male and has been demonstrated or assumed to have received the spermatophore from the male.) Nevertheless, even though a female cockroach may have passed several weeks in an apparently non-productive state, it is capable of sporadic outbursts of attractant, as shown in Fig. 2. During the non-productive phase the female does not attract the male and mating does not occur. On the other hand, during a shower of attractiveness mating may occur, even after a long period of inactivity (Fig. 2). We have known five females to mate for a second time. The effect of such an event is again to reduce or stop the production of attractant (Fig. 2). The probability (P), obtained from *Chi square* calculations, that the reduction in attractiveness is due to chance is as small after the second mating as it is after the first. Outcroppings of attractant productivity may occur after the second mating (Fig. 2), and, as this may take place quite early in a cockroach's maturity, we may infer that female *P. americana* may breed more than twice in nature. We have known one cockroach to mate for the second time when 33 weeks of age; and one to mate for the first time at 49 weeks. Multiple matings have been observed in other cockroaches, in *Blattella germanica* by KHALIFA (1950) and in *Diploptera punctata* (Eschtz) by STAY and ROTH (in press), who find it a frequent occurrence in *Diploptera*.

We have actually observed twenty-five female cockroaches mating, in all cases in a well-lit room. Time of copula was not less than 1 hr, nor more than 4 hr, the

average being 1 hr 44 min. To determine whether or not copulation has occurred, it is preferable to observe mating, instead of looking in the genital chamber for a spermatophore, this being less disturbing to the animal, and the effect of breeding alone being shown to have an adverse effect on production of the attractant, as seen in Fig. 1, and as indicated by $P = < 0{\cdot}0000001$. On the other hand, at first we were thwarted by the fact that *P. americana* tends to mate at night rather than in the day-time under our laboratory conditions of 8 hr of artificial light.

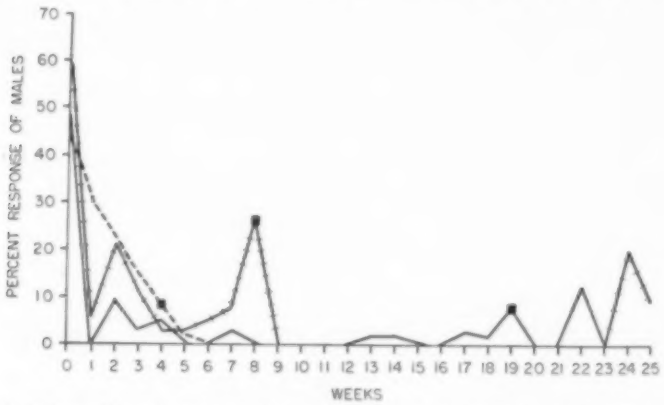

FIG. 2. Effect of breeding on weekly production of attractant by three different female *P. americana*, as determined by response of males to weekly accumulation of attractant deposited on paper by each female after being bred for the first and second time. ■ Bred for the second time.

This tendency prevails especially in the female, which often will not accept the male but rather retreats from its pursuit, only to mate later at night. Out of a total of 94 females aged 1–33 weeks which were exposed to males during a period of 16 hr at night, 56 (60 per cent) were bred. On the other hand, out of a total of 206 exposed to males during 8 hr of daylight only 26 (13 per cent) were bred. We found no seasonal variation in the percentages bred. The males, on the other hand, court actively during the day with females presented to them. There thus appears to be a difference between the sexes in periodism of sexual activity. A sex difference was not reported by MELLANBY (1940), GUNN (1940), or CLOUDSLEY-THOMPSON (1953) in the photoperiodism of the cockroach with respect to locomotor activity. Night matings, which occurred more frequently, were certified by finding the spermatophore in the genital atrium of the female the following morning. To make this observation we anaesthetized the females with CO_2 for 45 sec and examined them under the microscope while gently separating the genital segments with forceps. The effect of mating combined with these procedures affected production of attractant greatly. To determine how much of this effect was due to mating and how much to the other manipulations we compared two groups of females of similar age, one of which was mated, anaesthetized, and examined, and the second of which was exposed (but not mated), anaesthetized, and examined.

Thus, 24 females from 2 to 24 weeks of age were tested for their activity and found to arouse 39 per cent of the males on an average. They were then mated, and 1 week after evoked a response of only 7 per cent, from which point there was a continuous decline. A similar though less drastic decline occurred with the unmated group of 15 females. The difference was significant as indicated by P being $< 0{\cdot}002$. It could be deduced, therefore, that exposure, CO_2, and examination together had a depressing effect, though not as profound as when mating occurred.

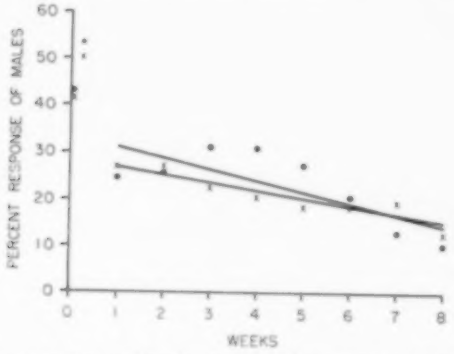

FIG. 3. Effect on attractant production induced by exposing females to males without breeding, CO_2, or examination. The probability of the deviations from the normal being due to chance $= > 0{\cdot}81$, indicating a lack of significant effect. ● Upper line = exposed when 2–6 weeks old. 15 roaches. × Lower line = normals, 5 roaches 2–6 weeks old at start.

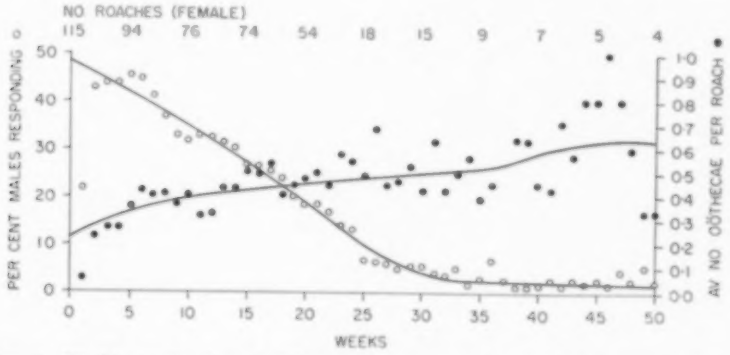
NO ROACHES (FEMALE)
115 94 76 74 54 18 15 9 7 5 4
PER CENT MALES RESPONDING
AV NO OÖTHECAE PER ROACH
0 5 10 15 20 25 30 35 40 45 50
WEEKS

FIG. 4. Weekly production of oöthecae and attractant in unmated *P. americana*. Note that as oöthecal production increases, production of attractant decreases. Both of these changes are accelerated by mating.

Further analysis of the three factors, exposure, CO_2, and examination, showed that when the cockroaches were exposed to males but did not mate, and were not anaesthetized or examined under the microscope, no significant depressing effect was found to have been caused by exposure to males alone (Fig. 3), as indicated by the fact that the probability, P, of the deviations from the normal being due to

chance was greater than 0·81. From the weekly examination of three groups of females we find no significant difference ($P = 0{\cdot}91$) caused by treatment with CO_2 for 45 sec.

We have not detected a periodism such as occurs with mammalian oestrus. Until interrupted by breeding, the attractiveness of the female is maintained fairly evenly and tapers off gradually (Fig. 4). Nor do we know the mechanism of depression of the attraction. Perhaps the seminal material exerts an inhibitory effect on the olfactant or it may absorb it. Or production of the attractant may be under hormonal control, and the seminal fluid, on entering the spermatheca, may induce a stoppage of endocrine function such as the apparent suppression of the stimulus of the corpus allatum by the increasing secretion of the prothoracic gland at the last instar (BODENSTEIN, 1953; WIGGLESWORTH, 1951; WILLIAMS, 1948); or such as oestrogens exert in inhibiting the liberation of the lactogens in mammals (BROWN, 1950); or the cessation of oestrus by conception.

There is a question whether the attractant is being constantly produced or whether it is stored, as neurosecretory material produced in the brain may be stored in the corpus cardiacum (SCHARRER, 1952), and hence, whether its apparent loss represents a decrease in productiveness, or is due to changes in a release mechanism such as has been suggested. The present data cannot answer this question.

Production of oöthecae

Periplaneta americana can reproduce parthenogenetically, although unmated females produce fewer oöthecae during their lifetime than do mated ones, as GRIFFITHS and TAUBER (1942) and ROTH and WILLIS (1956) have demonstrated. Hence we have a means of determining the possible relationship between oöthecal production and the attractant. Fig. 4 shows that attractant continues to be emitted during the course of normal oöthecal production. However, a gradual decline in attractant takes place while oöthecal production increases. Also, in Fig. 5, mating is shown to enhance the rate of oöthecal production sharply, whereas, as we have seen (Fig. 1), it depresses the attractant.

Copulation, in fact, increases the rate of oöthecal production, as indicated by the findings of GRIFFITHS and TAUBER (1942) and ROTH and WILLIS (1956), who found that females placed with males (and presumably mated) produced more oöthecae than unmated ones. The latter authors also found that fertilized female *P. americana* produced their oöthecae sooner than unmated females, and that this was also true of *Blatta orientalis*. We have examined the effect of mating at different periods of adulthood on the production of oöthecae by *P. americana* (Table 1 and Fig. 5).

Table 1 shows the average number of oöthecae produced per female during 5 weekly intervals and compares the performance of normal (unmated) females with that of females mated during successive 5 weekly periods of adulthood. The data show that all groups of mated females increased oöthecal production soon after mating, regardless of whether they were bred at 1–5, 5–10, 10–15, or

15–20 weeks after emergence. The increase is independent of any seasonal variation that may occur in oöthecal production such as noted by RICCI (1950) in *Blatta orientalis*. Fig. 5, in which the cumulative increases are plotted against the normal production, represented by the base line, shows that the increase is

TABLE 1—EFFECT OF MATING ON OÖTHECAL PRODUCTION BY *P. americana*

	Number of oöthecae per female during 5 weekly periods								
	1–5	5–10	10–15	15–20	20–25	25–30	30–35	35–40	40–45
54 unmated	1·3	2·3	2·2	3·1	3·3	2·7	3·0	2·8	2·6
36 mated during weeks 1–5	2·2	4·5	4·1	4·5	3·3	4·8	3·2	3·2	2·3
8 mated during weeks 5–10	1·0	2·9	7·4	6·8	5·8	4·4	3·7	3·0	
9 mated during weeks 10–15	1·8	2·0	2·5	3·1	4·8	3·8	2·8	2·6	2·0
9 mated during weeks 15–20	1·7	2·6	1·6	3·9	5·1	4·8	2·2	2·6	

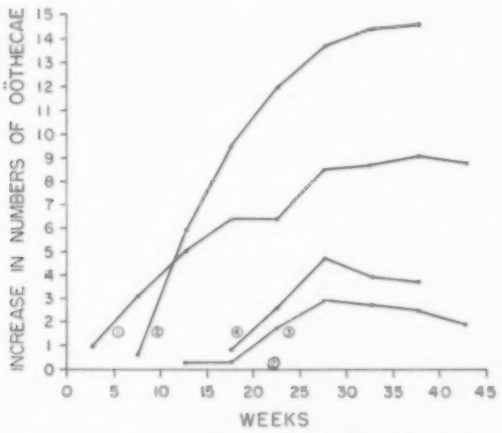

FIG. 5. Increase in oöthecal production per roach induced by breeding. (1) bred at 0–5 weeks; (2) bred at 5–10 weeks; (3) bred at 10–15 weeks; (4) bred at 15–20 weeks; (C) base line = unmated controls. Number of roaches at start: (1) 36; (2) 8; (3) 9; (4) 9; (C) 54.

not haphazard but on the contrary continuous, and it is manifested most strongly by the groups mated at an early age. The oviposition stimulus lasts throughout the life of the cockroach and attains its maximal effect several weeks after impregnation.

The sharpness and extent of the rise among those insects mated at 5–10 weeks after emergence indicates that the optimal time for the stimulus to be effective may be during this period.

The stimulus of breeding is not always sufficient to accelerate the production of oöthecae, however. For example, two of our cockroaches, neither of which had produced an oötheca, were mated at 2 and 4 weeks of age, respectively. They had been strongly attractive and were demonstrated to have received the spermatophore from the male, yet neither cockroach produced an oötheca during its lifetime; at the same time, attractant yield suffered the usual reduction. The connexion between attractance and oöthecal production is obscure; its study may well unfold an important endocrine relationship.

There may be significance in the lag observed in Fig. 5 (3) among the cockroaches mated during the 10–15 week period. This is suggested also by the decline in the rate of production of oöthecae that takes place at about this time in all groups of insects observed (including several groups of unmated cockroaches which provided the data for the composite base line of Fig. 5), except those mated during the 5–10 week period.

Egg maturation is under the control of hormones originating in the corpora allata and in the neurosecretory cells of the brain (SCHARRER, 1952; THOMSEN, 1952). We may suppose, therefore, that fertilization stimulates one or both of these centres to produce greater amounts of hormone. On the other hand, since the corpus cardiacum acts as a reservoir for secretions of the neurosecretory cells (SCHARRER, 1952), the effect of fertilization may rather be to cause a greater rate of release of the hormones stored in this body. It is entirely possible, however, that the contents of the spermatophore supply a gonadotropic or even a gonadal hormone which acts directly or indirectly on the ovaries. The stimulus, on the other hand, may be partly nervous. Furthermore, the contents of the spermatophore may supply nutrients which stimulate egg maturation, either by being absorbed or by fertilization. The view that the state of fertility of the ovum may be a factor in regulating ovulation is supported by the findings of ROTH and WILLIS (1956) that among the progeny of mated and unmated female *Blatta orientalis*, failure to ovulate occurred in the following order: in 2 out of 30 mated females; in 10 out of 20 unmated females; and in 25 out of 34 unmated females from parthenogenetic parents. On the other hand, when parthenogenetic females were mated there was a very great increase in oöthecal production which exceeded even the normal.

The effect of radiation

The effect of radiation on the production of attractant was studied to determine (a) how low a dose of non-lethal radiation could affect the production of attractant, and (b) whether, in view of the possibly significant relationship between production of attractant and of oöthecae, a connexion between the two might be further indicated by the action of the radiation on the ovaries. For this purpose sets of 12 two-week-old females were put into 5 × 12 in. polyethylene bags, the necks of which were then tied about polyethylene ventilating tubes, and irradiated with 1000, 2500, 5000, and 50,000 r by means of a 2 MeV Van de Graaff electron accelerator and then returned to their battery jars. At weekly periods the papers

lining these jars were replaced with fresh papers and tested by bioassay against a standard solution of attractant. The assay was done by using a test of two rounds in the first of which the entire paper was folded and presented to the male cockroaches. For the second round, half of the paper was used after it had been cut so as to halve the apparent soilage; halves made in this way were found by assay to be approximately equal in potency. Table 2 summarizes our findings.

TABLE 2—THE EFFECT OF CATHODE RAYS ON SURVIVAL AND ON OÖTHECAL AND ATTRACTANT PRODUCTION BY FEMALE *P. americana*

	Days								
	3	5	6	7	14	21	28	35	42
	No. dead								
1000 r	0	0	0	0	0	0	0	0	0
2500 r	0	0	0	0	0	1	2	3	3
5000 r	0	0	1	2	3	6	10	10	11
50,000 r	7	10	10	10	12				
Controls	0	0	0	0	0	0	0	0	0
	No. of oöthecae								
1000 r				0	0	0	0	0	0
2500 r				0	0	0	0	0	0
5000 r				0	0	0	0	0	0
Controls				5	11	17	24	30	36
	Rel. potency*								
1000 r				0·29	0·9	0·86	2·3	1·6	1·8
2500 r				0·38	0·83	0·68	0·2	1·3	2·9

* Attractant yield (determined by bioassay of papers from jars against a standard solution) given in terms of paper from control jar as having a value of 1.

Doses of 50,000 and even 5000 r were too lethal to permit continuous tests for attractant and oöthecal production. However, a dose of 1000 r was not lethal to animals observed over a period of several weeks, and 2500 r proved to be about the lethal threshold. A most striking fact is that the irradiated cockroaches did not produce any oöthecae during the 6 week period of observation, whereas the unirradiated controls produced 36 oöthecae. Attractant production also was adversely affected. No great difference was shown in the effects of 1000 and 2500 r; at the end of the first week after irradiation the cockroaches of both groups had emitted about one-third as much attractant as the controls and continued to show the damaging effect of radiation for another 2 or 3 weeks. A remarkable reversal then occurred in production of attractant, so that at 4 weeks the relative activity of the females irradiated with 1000 r was 2·3, while at 6 weeks the relative activity of those irradiated with 2500 r was 2·9. Presumably recovery was retarded in the latter group because of the greater damage done by the larger dose. It is

interesting that the recovery of attractant production among these cockroaches should overshoot the normal mark. In view of the fact that oöthecal production was destroyed, or at least totally inhibited by irradiation, while in normal females there is an inverse relationship between oöthecal production and attractant production, these findings suggest that the over-recovery of attractance in the irradiated insects may be due to the destruction of a controlling mechanism associated with oöthecal production.

SUMMARY

A correspondence has been shown to exist between the production of attractant and the mating period of *Periplaneta americana*. The attractant is produced principally by virgin females and, sporadically, by mated females, and is conducive to mating; however, mating depresses the production of attractant.

Manipulation of the female can depress the production of attractant.

Production of attractant normally decreases with age and as oöthecal production increases.

Mating induces an increase in oöthecal production, especially among younger cockroaches.

Cathode-ray irradiation damages oöthecal and attractant production. With oöthecal production totally inhibited by irradiation, the females recovered their capacity to produce attractant and exceeded the normal yield.

A relationship is indicated between attractant production and the processes which regulate ovulation.

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ELECTROPHYSIOLOGICAL STUDIES OF ARTHROPOD CHEMORECEPTION—II

RESPONSES OF LABELLAR CHEMORECEPTORS OF THE BLOWFLY TO STIMULATION BY CARBOHYDRATES*

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Abstract—The effects of 23 carbohydrates were tested upon the labellar chemoreceptors of the blowfly, *Phormia regina* Meigen. Results of electrophysiological analysis of receptor activity in single labellar hairs were compared with behaviour evoked by the same chemicals when applied to single hairs of intact flies, as studied by DETHIER. There is a close correlation between the presence of afferent impulses from the *S* receptor and occurrence of a feeding response (proboscis extension). Absence of a feeding response may be caused by failure of the chemical to stimulate the *S* receptor, by strong stimulation of the *L* receptor, or by inactivity of both *S* and *L* receptors. The distinction between "repellent" and "inhibitory" compounds has some basis in terms of the effects of the two categories of compounds upon the labellar receptors. Since the *S* and *L* receptors do not operate completely independently, however, any chemical which strongly stimulates one receptor cell appears to always inhibit the other receptor cell. This mechanism is believed to enhance the contrast between "acceptable" and "unacceptable" stimuli activating the labellar hairs, and consequently increases the effectiveness of the peripheral discrimination mechanism.

PREVIOUS electrophysiological study of labellar chemoreceptors of flies supported the hypothesis of a peripheral discrimination mechanism for sugars and non-sugars (HODGSON and ROEDER, 1956). Thus, when a series of salts, sugars, acids, and alcohols were tested on the labellar chemosensory hairs of *Phormia*, one of the receptor cells in the hair (the *S* receptor) was stimulated by sugars, and another receptor cell (the *L* receptor) was stimulated by the non-sugars. Since *Phormia* feeds upon sugars, it was postulated that stimulation of the *S* receptor mediated feeding responses, and stimulation of the *L* receptor mediated so-called "rejection" responses of the fly. The possibility of such a peripheral discrimination mechanism for compounds acceptable or unacceptable as food had been suggested earlier on the basis of behavioural studies on flies (DETHIER, 1953), and the specialization of the *L* and *S* receptors was interpreted as being the basic physiological mechanism underlying the behavioural effects.

Further observations on the feeding behaviour of flies have raised a number of additional questions about the relationship between receptor activity and behaviour of the whole animal. DETHIER (1955) and DETHIER, EVANS and RHOADES (1956)

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reported that some carbohydrates never evoked feeding responses when applied to single labellar hairs, and a correlation was noted between low thresholds of feeding behaviour and an α-D-glucopyranoside linkage in the stimulating sugar molecules. It was also found that certain chemicals actually inhibited behavioural responses—e.g. mannose added to fructose solution elevated the threshold of response to fructose, and salts of heavy metals caused prolonged periods of insensitivity to normal stimulation by sugars. GALUN and FRAENKEL (1957) found that certain pentoses were repellent or even toxic to flies. While the latter experiments did not test sugars directly on individual labellar hairs, they did involve behavioural responses which clearly depended upon chemical stimulation of the mouthparts of flies, and hence should have a bearing upon the physiology of the labellar receptors.

The object of the present work was to determine in what ways the original concept of a direct relationship between functions of the *L* and *S* receptors and the fly's feeding behaviour should be extended or modified to account for these data. Specifically, answers to the following questions were sought: (1) Do sugars which fail to evoke feeding responses fail to stimulate the *S* receptor, as would be expected from the initial hypothesis regarding the functions of that cell? (2) Can different thresholds of behavioural reactions to various sugars be correlated with different frequencies of afferent impulses from the *S* receptor? and (3) How do sugars and heavy metal salts with repellent or inhibitory behavioural effects act upon the primary labellar chemoreceptors?

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MATERIALS AND METHODS

The experiments were performed upon the longest chemosensory hairs on the labellum of the blowfly *Phormia regina* Meigen. Afferent impulses in the chemoreceptor cells associated with these labellar hairs were recorded by a technique which has been described in detail elsewhere (HODGSON, LETTVIN and ROEDER, 1955; HODGSON and ROEDER, 1956). Since some electrolyte must be present in the stimulating-recording electrode, all of the sugars were administered in 0·1 M NaCl solutions. This concentration of salt is high enough to avoid certain aberrant effects of very dilute salt solutions (HODGSON and ROEDER, 1956), yet it does not stimulate enough impulses from the *L* receptor to obscure the patterns of *S* spike potentials which constitute the data actually desired.

Each test was run on at least four different labellar hairs, located on at least two different flies. A hair was first exposed to 0·1 M NaCl alone to make sure that the *S* fibre was not responding to solvent solutions, and then a 0·2 M concentration of the test sugar mixed in 0·1 M NaCl was applied. If *S* spikes were present in the receptor activity which then resulted, a positive response of the *S* receptor was recorded as the effect of that particular test sugar. The few cases where results were not consistent in all hairs tested are noted at appropriate points below.

Comparisons of the stimulating effectiveness of two sugars, both of which stimulate the *S* fibre, required determination of their effects on the same hair because of threshold differences between the hairs. In this case the two sugars were tested alternately on the same hair, with two rinses of 0·1 M NaCl applied during a 4 min interval allowed for disadaptation of the receptor between tests. Quantitative measurements of receptor cell activity were made by counting the number of afferent impulses during a 1 sec interval. The initial $\frac{1}{10}$ sec during stimulation was disregarded in the records because of troublesome artifacts common during that time (HODGSON and ROEDER, 1956), and the second immediately

following that interval was used for comparisons of the quantitative data in all cases. This procedure, used with the usual methods of statistical analysis, gave the most reproducible results of several procedures tried in preliminary tests, although the irregularity which characterizes the pattern impulses of the *S* receptor always increased the variance in any quantitative comparisons of sugar effects.

RESULTS

The results of tests with 23 sugars or related compounds are shown in Table 1, where they are compared with the behavioural responses to the same compounds reported by DETHIER (1955). In Table 1, + designates the presence of *S* spikes in the electrical recording of activity during stimulation with a particular sugar, or (in the case of the behavioural data) it designates a proboscis extension response

TABLE 1—COMPARISON OF RECEPTOR ACTIVITY AND BEHAVIOURAL RESPONSES OF INTACT FLIES EVOKED BY STIMULATION OF SINGLE LABELLAR HAIRS WITH CARBOHYDRATES

Chemical	Behavioural response (from DETHIER, 1955)	Response of *S* receptor	No. of hairs tested electrophysiologically
Hexoses			
D-glucose	+	+	6
D-fructose	+	+	11
L-sorbose	+	+	4
D-galactose	+	+*	8
D-mannose	−*	+*	6
Pentoses			
D-arabinose	+	+	16
L-arabinose	+	+	16
D-xylose	+	+*	8
L-xylose	+	+*	8
D-ribose	−	(inhibition)	8
2-desoxyribose	−	(inhibition)	8
D-lyxose	−	(inhibition)	8
Di-, tri-, and poly-saccharides			
Sucrose	+	+	76
Cellobiose	−*	−	4
Lactose	−	−	4
Melezitose	+	+*	4
Raffinose	+	+*	4
Xylan	—	—	4
Glycogen	—	—	4
Polyhydric alcohols			
Sorbitol	−	−	8
Ducitol	−	*L* 75% of cases	8
Mannitol	−	*S* 25% of cases	8
Inositol	+	+	8

* Indicates a compound effective on groups of labellar hairs but not on single hairs in behavioural tests, or one which evokes only a few *S* impulses in electrophysiological tests.

when a solution of the sugar is applied to the tip of a single labellar hair. A comparison of the receptor responses and the behavioural responses shows that only those sugars which stimulate the *S* receptor evoke a feeding response (proboscis extension). Record *A* of Fig. 1 shows the effect of a weakly stimulating sugar (raffinose) upon a labellar hair, and record *B* shows the effect of a highly stimulating sugar (fructose) applied to the same hair.

None of the carbohydrates (except for polyhydric alcohols) was observed to stimulate the *L* receptor, but rather their application caused a decrease in the frequency of impulses from the *L* receptor, as compared with the *L* activity produced by the solvent alone. In general, the greater the number of impulses from the *S* receptor, the less the number of impulses from the *L* receptor, as might be illustrated by a comparison of records *A* and *B* in Fig. 1, in which the *L* spikes are obviously fewer during application of fructose which is more stimulating to the *S* receptor. Plots of the data from tests in which varying proportions of NaCl and sucrose were applied as mixtures to the same hair showed, however, that an inverse relationship between the frequencies of *S* and *L* spikes, although it may be roughly approximate, is not really a consistent relationship throughout the whole range of concentrations of salt or sugar usually tested. Some interpretations of this phenomenon are discussed below.

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With the exception of arabinose, the pentoses caused either very little stimulation of the *S* receptor or inhibition of both *L* and *S* activity. Ribose, 2-desoxyribose, and D-lyxose produced the most pronounced inhibitory effects, as illustrated by comparing records *C* and *D* of Fig. 1. In record *C* fructose evokes many *S* spikes, yet in record *D* ribose applied to the same hair evoked only the one *S* spike shown in the photograph, and the activity of the *L* fibre is decreased. The ribose effect was easily reversed by a rinse with 0·1 M salt solution and a 4 min disadaptation period.

Perhaps the most striking correlation between receptor activity and behavioural responses was observed in tests with the polyhydric alcohols. Although all the four polyhydric alcohols tested have the same empirical formulae, sorbitol, dulcitol, and mannitol stimulated the *L* receptor predominantly, with weak stimulation of the *S* receptor in only 25 per cent of the cases when dulcitol and mannitol were applied. Inositol stimulated the *S* receptor predominantly, and behavioural tests (DETHIER, 1955) showed that inositol alone in the group of compounds evoked a feeding response. The results with the polyhydric alcohols are illustrated by a comparison of records *E* and *F* of Fig. 1, which show the effects of dulcitol and inositol, respectively, applied to the same labellar hair.

Quantitative comparisons of the effects of various sugars and inhibitors were made only for certain selected pairs of sugars because of the difficulty of getting enough data from a single preparation to permit adequate statistical treatment. Typical results are shown in Table 2. Each set of data is derived from photographs of receptor activity in a single labellar hair. The variations in sensitivity of different hairs did not make combinations of data from many hairs feasible. A difference greater than twice its standard error in four repetitions of a test was considered

significant. The results show that effects of very stimulating sugars (fructose, sucrose) and very weakly stimulating sugars (xylose, raffinose) are significantly different when compared on the basis of the frequencies of *S* spikes evoked (see also records *A* and *B* of Fig. 1). This method could not, however, demonstrate a significant difference between the stimulating effectiveness of the isomers D-arabinose and L-arabinose, which differ in their stimulating effectiveness when tested behaviourally. Additive effects could be demonstrated for D-arabinose and L-arabinose (Table 2) but not for the other combinations tested, presumably because one sugar of the pair was usually very weakly stimulating or else was maximally stimulating to the receptor so that in either case any additional sugar was without significant effect. Significant influences of mannose or sorbose upon the fructose and sucrose responses could not be shown, although such effects might be expected from the behavioural data (DETHIER, 1955 ; DETHIER, EVANS and RHOADES, 1956).

Effects of salts of heavy metals were tested by applying the salt, prepared in Pringle's insect saline, for 1 min and then testing the same labellar hair with a

TABLE 2—EFFECTS OF VARIOUS TREATMENTS UPON ACTIVITY OF *S* RECEPTOR

Each set of data derived from counts of *S* spikes during 1 sec, beginning $\frac{1}{16}$ sec after administration of the stimulus. Mean values of counts from four replicates of each test, standard errors, are given. An asterisk indicates that the difference between means for that set of data is greater than twice the standard error of the difference, indicating a significantly different effect of the treatments upon *S* impulse frequency.

Compound	Mean no. of *S* spikes *S.E.*	2 × *S.E.* of difference in means
Sucrose	50 ± 11	26*
Xylose	10 ± 8	
Fructose	57 ± 13	28*
Raffinose	9 ± 6	
D-arabinose	21 ± 10	34
L-arabinose	17 ± 11	
D-arabinose plus L-arabinose	62 ± 12	30, 30*
Fructose	60 ± 19	44
Mannose plus fructose	55 ± 11	
Sucrose	36 ± 8	24
Mannose plus sucrose	42 ± 9	
Fructose	73 ± 12	38
Sorbose plus fructose	60 ± 15	
Sucrose	40 ± 8	26
Sorbose plus sucrose	35 ± 10	
Sucrose	54 ± 13	36
Sucrose plus D-ribose	47 ± 12	

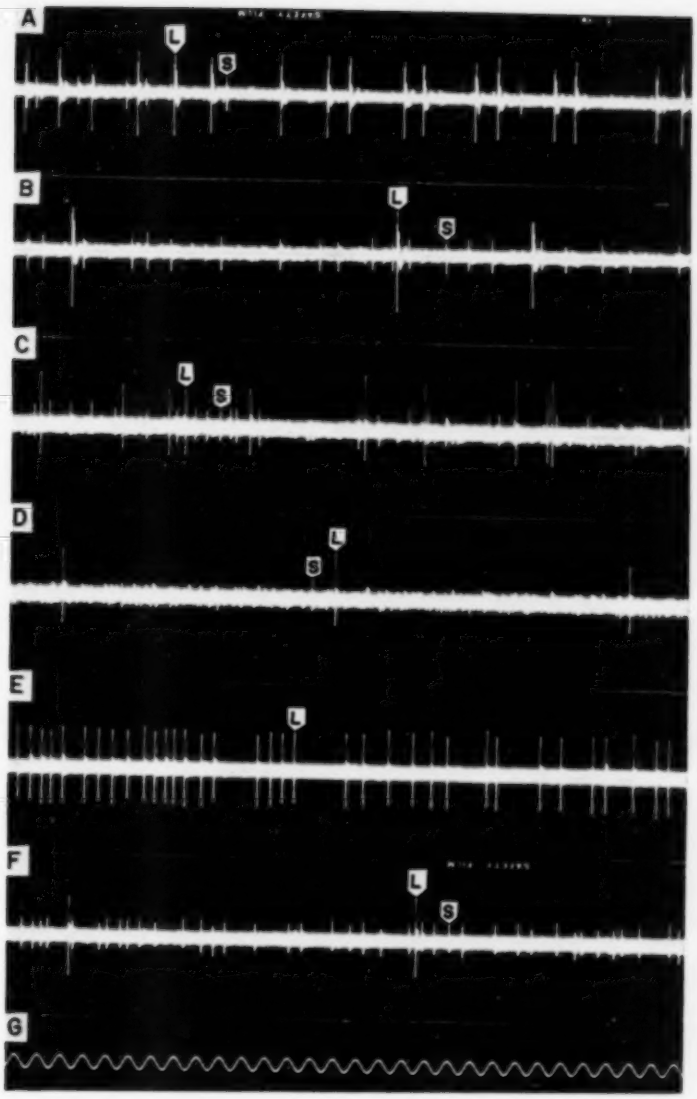

FIG. 1. Receptor activities recorded during one second, beginning $\frac{1}{10}$ sec after application of carbohydrates. *A* = raffinose, *B* = fructose applied to the same hair ; *C* = fructose, *D* = ribose applied to the same hair ; *E* = dulcitol, *F* = inositol applied to the same hair ; *G* = time-base 100 cycles/sec. The labels *S* and *L* indicate the spike potentials from the two receptor cells. All records have been tape recorded but not filtered.

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mixture of 0·25 M NaCl and 0·2 M sucrose. Although a 1 min exposure to a saline control solution has no effect on later records, all of the heavy metal salts did. $HgCl_2$ and $FeCl_2$ at 5×10^{-4} M concentrations caused a decrease or grouping of *L* spikes and eliminated the *S* spikes. Higher concentrations killed the receptor cells within a few minutes. With $CuCl_2$, $BaCl_2$, $CaCl_2$ at comparable concentrations, the afferent impulses were definitely abnormal, showing decreased frequencies of *L* spikes or grouping, but *S* impulses were still recorded. These data possibly explain the lack of behavioural responses to sucrose after $HgCl_2$ (DETHIER, 1955) as being due to elimination of *S* receptor activity but indicate that $CuCl_2$, $BaCl_2$, and $CaCl_2$, while producing definite effects upon the receptors, are not differentially eliminating the *S* impulses.

DISCUSSION

The present data indicate a consistent correlation between afferent activity in the *S* fibre and a feeding response of the intact animal. The significant quantitative differences in frequencies of *S* impulses evoked by various sugars also support the hypothesis that an α-D-glucopyranoside linkage in the stimulating molecule increases its action upon the *S* receptor and thereby causes a low threshold of behavioural response. The absence of a feeding response, however, can result from several mechanisms: the *S* receptor may not be stimulated while the *L* receptor is minimally active under the usual test conditions (exactly as when the solvent alone is applied), the *L* receptor may be strongly stimulated while the *S* receptor is inactive (as when dulcitol is applied), or the activity of both receptor cells may be practically absent (as with ribose). It would be difficult to identify any one of these mechanisms by behavioural tests alone, although DETHIER, EVANS and RHOADES (1956) have differentiated between the effects of "repellence" and "inhibition" in recent behavioural tests. As the two terms are defined by these workers and might be used in connexion with the labellar hairs, a repellent chemical would be expected to stimulate the *L* receptor, while an inhibitor chemical would lower the frequency of afferent impulses from one or both receptors. Thus the repellency of ribose noted by GALUN and FRAENKEL (1957) in behavioural tests might be considered a case of inhibition of receptor cell activity, while sodium chloride would be considered a true repellent because it stimulates the *L* receptor. However, no significant effect of ribose on the *S* fibre response could be demonstrated (Table 2). The "inhibition" in this case seems to be a decrease in the frequency of *L* spikes and a virtual lack of stimulation of the *S* fibre. A theory facilitating the predictive distinction between repellent and inhibitory effects, based upon molecular structure of the stimulants, will have to await further studies, both behavioural and electrophysiological.

While the differentiation of "inhibition" and "repellency" in both behavioural and electrophysiological experiments undoubtedly marks a real advance towards a more precise description of the chemoreceptive mechanism, the distinction between the two processes is complicated by the fact that they probably never operate independently. Presence of *S* impulses is characteristically accompanied

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by a decrease in *L* impulses in our records, and stimulation of the *L* fibre is associated with a decrease in the frequency of *S* spikes. Consequently, a "repellent" stimulating the *L* receptor would act at the same time as an "inhibitor" for the *S* receptor, and a stimulant for the *S* receptor would act as an "inhibitor" for the *L* receptor. The mechanism of this apparent interaction is unknown, although the absence of a consistent inverse relationship between the frequencies of *S* and *L* impulses indicates that the facts cannot be explained simply as the result of partial depolarization of one receptor unit by electrotonic spread from the more active adjacent unit. The actual mechanism is currently unknown, but its eventual behavioural effect must be an increase in the contrast between chemicals acceptable and unacceptable to the insect as food. A somewhat similar phenomenon has been observed in the eye of *Limulus*, where adjacent ommatidia often inhibit one another mutually, thereby enhancing visual contrast (HARTLINE, WAGNER and RATLIFF, 1955).

Some quantitative discrepancies between the behavioural and electrophysiological results do exist. Thus, it is not always possible to interpret behavioural threshold differences solely in terms of the frequencies of the *S* spike potentials in these experiments. This is not surprising in view of certain necessary differences in the techniques of the behavioural and the electrophysiological experiments. Behavioural thresholds are determined by applying the sugars in aqueous solutions. Electrophysiological measurements had to be made with some electrolyte also in the solution, and since the *L* and *S* receptors do not operate completely independently, this may explain why the concentrations of sugars used in the electrophysiological tests had to be higher than those in the behavioural tests in order to obtain numbers of *S* spikes sufficient for statistical analysis. Another factor contributing to the difficulty of establishing statistically significant differences between frequencies of *S* impulses is the irregularity of these impulses. Some grouping of the *S* spikes, occasional periods of inactivity, etc. seem to be characteristic of the *S* receptor (HODGSON and ROEDER, 1956) and these features greatly increase the variance of most counts of *S* impulses during the limited intervals. The electrophysiological method in its present stage of development can demonstrate the basis for the larger differences between stimulating and non-stimulating chemicals, but is not as sensitive as the behavioural tests in comparing thresholds which are more closely matched. For this reason, although the electrophysiological experiments give direct access to many types of information otherwise difficult to obtain, their maximum usefulness in many cases will be realized when they are employed in combination with behavioural tests.

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STUDIES ON THE DEGENERATION OF THE FLIGHT MUSCLES OF ALATE APHIDS—I

A COMPARATIVE STUDY OF THE OCCURRENCE OF MUSCLE BREAKDOWN IN RELATION TO REPRODUCTION IN SEVERAL SPECIES

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Abstract—When alate aphids of several species were allowed to settle down on their host plants, their flight muscles began to break down within a few days. The onset of muscle degeneration could be delayed by preventing the aphids from settling down, either by denying them their host altogether or by leaving them on a poor host in darkness for several days.

The reproductive capacity of alatae before they lost the ability to fly varied for different species. Embryo development was arrested in alatae of all the species that were studied after the end of the teneral condition following the final ecdysis, and was resumed when they settled down on a new host. Some species of aphids contained a number of fully formed embryos at the time embryo development was arrested, and these plus a few additional embryos whose development had been completed after the aphids settled down were born before the ability to fly was lost. In other species the young alatae contained only rudimentary embryos that required several days to develop to the stage at which they were normally born; in these species no larvae were born until the muscles had begun to degenerate and the ability to fly was lost. Other species fell between these two groups and in one species, *Macrosiphum euphorbiae* (Thomas), whether or not the aphids reproduced at all before losing the ability to fly depended upon their size.

The ability of alatae of *Aphis fabae* Scop. to engage in long flights of 1 hr or more was retained for as long as the aphids retained the ability to fly.

INTRODUCTION

THE first record of the histolysis of the flight muscles of an insect was made by JANET (1907), who showed that the voluminous flight muscles of queen ants degenerated after the nuptial flight. A few years later FEYTAUD (1912) found that flight-muscle breakdown occurred in queen termites, and since then it has been recorded in some Diptera (MERCIER, 1924, 1928; HOCKING, 1952), Coleoptera (JACKSON, 1933, 1953), aquatic Hemiptera (POISSON, 1924), and aphids (JOHNSON, 1953), and it probably also occurs in Dermaptera (MERCIER and POISSON, 1923). In ants, termites, and the mosquito probably *Aëdes communis* muscle breakdown appears to be associated with the development of eggs, but this does not seem to be the case in beetles (JACKSON, 1933).

* This work was done at Rothamsted Experimental Station, Harpenden, Herts., England.

The flight muscles of alate aphids, like those of ants, termites, and Diptera, degenerate after the insects have settled down to a sedentary life following a dispersal flight or flights. BROADBENT (1949) showed that some species of aphids retained the ability to fly for a few days after they had produced some larvae. In the present study the reproductive capacity of a number of species of aphids before they lose the ability to fly is compared. An account of the histology and physiology of muscle breakdown will be given in a later paper.

MATERIALS AND METHODS

The aphids used were alate *alienicolae* and were mostly obtained from natural infestations of their host plants as indicated in Table 1. Small aphids were avoided, and the aphids used were from healthy colonies and in each of the experiments were of more or less uniform size. They were collected while they were in the teneral condition following the final ecdysis and left for about 20 hr on leaves of their host plant in darkness; during this time they remained on the leaves, but did not reproduce. When they were taken for the experiments they had all spent a few additional hours on the leaves after their wings and cuticle had hardened.

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The aphids were then allowed a short flight in a glasshouse and placed on suitable fresh host plants. This treatment released the settling response (JOHNSON, 1957), and the aphids then remained on the plants feeding and reproducing. The methods used for handling the aphids and allowing them tethered and untethered flight were the same as described in JOHNSON (1957).

The larvae produced each day were counted and removed, and the alatae were tested to see if they were still able to fly either by dropping them in a brightly illuminated chamber or by attaching them to pins and stimulating them with a light puff of air. Some of the aphids which no longer gave any indication of being able to fly were dissected or sectioned and their flight muscles were examined for signs of histolysis.

EMBRYO DEVELOPMENT IN ALATE APHIDS

At metamorphosis the ovaries of alate aphids are well developed and each of the ovarioles contains a number of embryos in various stages of development. The number of ovarioles and the number of oöcytes which have been extruded by the last moult varies with different species. In *Aphis fabae* Scop. there are typically twelve ovarioles each containing about six embryos. Growth of the embryos continues during the teneral period after the last ecdysis and for several hours after the cuticle and wings have hardened, and it is then arrested. The stage that the largest embryos reach before growth is arrested varies for different species. In some species each of the ovarioles contains one and sometimes two fully formed embryos (e.g. *Sappaphis mali*); in others the largest embryos are less than half the size to which they grow before being born (e.g. *Tuberolachnus salignus*). Once arrested, embryo development is not resumed until the aphids settle down to feed on a new host plant. If they are kept off their host plant, or if they are kept on a host plant from the final ecdysis in darkness and without having engaged in any

locomotor activity, it is not resumed, and further development does not take place until the aphids settle down, such as after a flight or other activity (JOHNSON, 1957).

REPRODUCTIVE CAPACITY OF DIFFERENT SPECIES BEFORE LOSS OF ABILITY TO FLY

The numbers of larvae produced by young virginoparous alatae of several species of aphids in the first few days after settling down on their respective host plants are given in Table 1. The response of the aphids when attempts were made to stimulate them to fly, and the conditions of the flight muscles of aphids which could not be brought to fly, are also indicated.

TABLE 1—REPRODUCTIVE CAPACITY OF DIFFERENT SPECIES BEFORE DEGENERATION OF THE FLIGHT MUSCLES

Species	Original host	New host	Average number of larvae born and muscle condition each day *after* settling down				Total larvae born before loss of ability to fly		Number of aphids
			First day	Second day	Third day	Fourth day	Average	Range	
Sappaphis mali Ferr.	Apple	Plantain	17·0, *F*	2·1, *F*	1·8, *M*	—	17·6	16–20	11
Aphis fabae Scop.	Broadbean	Broadbean leaves	10·4, *F*	3·5, *F*	1·9, *M*	1·0, *H*	15·8	10–21	79
Brevicoryne brassicae (L.)	Cabbage	Cabbage seedlings	9·0, *F*	1·8, *F*	1·2, *M*	1·2, *H*	12·5	11–16	8
Myzus persicae Sulz.	Turnip	Turnip seedlings	4·3, *F*	2·2, *F*	2·2, *M*	2·1, *H*	9·3	6–13	14
Macrosiphum euphorbiae (Thomas)	*Aquilegia* leaves	*Aquilegia* flowers	0, *FM*	3·5, *MH**	3·0, *H*	—	3·5*	0– 6	10
Megoura viciae Kalt	Broadbean	Broadbean	0, *FM*	0, *M*	0, *H*	4·5, *H*	0	—	10
Tuberolachnus saligna Gmelin	Willow	Willow	0, *F*	0, *M*	0, *M*	0, *H*	0	—	4
Cinaropsis pinicola Kalt	Pine	Pine	0, *F*	0, *M*	0, *H*	2·0, *H*	0	—	2

F — When tested the aphids were found to be capable of flight.
M — Aphids would not fly when stimulated ; frequently there was no histologic evidence of muscle breakdown.
H — Flight muscles had begun to break down.
* See p. 252.

Twenty-four hours after they had settled down all of the aphids except three *M. euphorbiae* and six *M. viciae* were still able to fly. After 2–3 days the flight muscles of alatae of all species had begun to degenerate. Coincident with this was a distension of the abdomen which in some species (e.g. *M. viciae*) increased to as much as three to four times its original size (see Plate 1).

In the first four species the aphids contained a number of fully developed embryos that were born soon after they settled down on the plants (JOHNSON, 1954). Further embryo development occurred after settling down, and more embryos reached the fully developed state and were born over the next 48 hr

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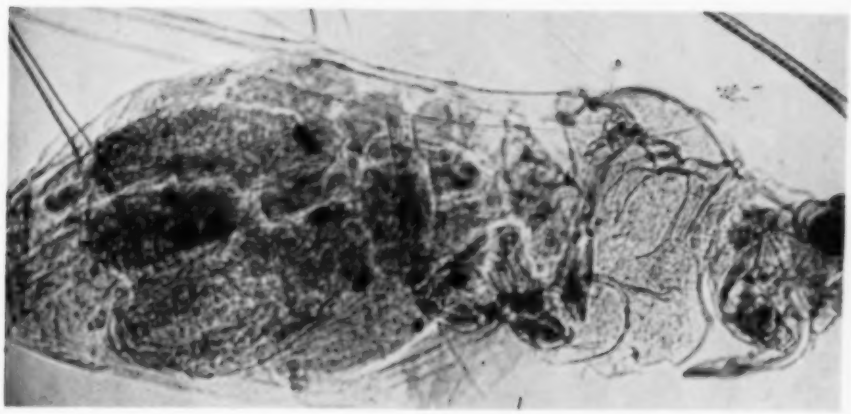

PLATE 1

FIG. 1. Alatae of *Brevicoryne brassicae* (L.). Left, young aphid at time of settling down after flight. Right, aphid which has been settled down feeding for 5 days and has lost the ability to fly.

FIG. 2. *Acrythosiphon pisum*. Young aphid with intact muscles and unswollen abdomen. Fixed in Carnoy's, cleared in methyl benzoate, unstained, cut in half.

FIG. 3. *Acrythosiphon pisum*. Aphid which has been settled down on a host plant for 5 days. The flight muscles have degenerated and disappeared and the abdomen has become greatly distended. Prepared in the same way as Fig. 2.

before the aphids lost the ability to fly. Different numbers of larvae were produced by the different species, but in all species the aphids could not be brought to fly after they had been settled down for more than 2 days.

In the last three species no larvae were born before the aphids had lost the ability to fly and the muscles had begun to degenerate. Embryo development was resumed after the aphids settled down to feed, but the largest embryos required 3–4 days' growth before reaching a stage when they could be born. The aphids could not be stimulated to fly after they had been settled down for more than 1 day.

Alatae of *M. euphorbiae* were intermediate between these two groups. The young alatae of this species contained a number of what appeared to be fully formed embryos, but when the aphids settled down these increased considerably in size and became more rounded before being born. The first births frequently did not occur until the aphids had been settled down for about 24 hr. However, if the aphids were prevented from feeding, as by cutting off their stylets, larvae were frequently born within an hour and without first increasing in size. The production of these small larvae also occurred when intact aphids were released on less attractive hosts (such as mature leaves of potato) after a few hours' activity in a glass tube.

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The embryos of *M. viciae* were less developed than those of *M. euphorbiae*, and after settling down after flight the aphids fed for 3–4 days and became greatly distended before larvae were born. But although extensive embryo development normally occurred before any were born, like *M. euphorbiae*, alatae of *M. viciae* can under certain conditions deposit smaller less developed larvae before losing the ability to fly. One alate aphid which was confined in darkness without flight for 4 days did not reproduce until it was allowed a short flight and put on a leaf; it then settled down and gave birth to three thin emaciated-looking larvae with bent legs within a few hours and before the 3–4 days' feeding that normally intervenes before any births occur.

EFFECT OF SIZE ON REPRODUCTIVE CAPACITY BEFORE LOSS OF ABILITY TO FLY

There is a considerable variation in the size of aphids produced on different plants within their host range, and on different stages of growth of the same host plant. Aphids produced in old colonies on plants that are wilting or dying are frequently very reduced in size. High temperatures can also cause reduced aphid size (KENTON, 1956). An experiment was conducted to determine the effect of size on the reproductive capacity of alatae of a single species before they lost the ability to fly.

Alatae of *Macrosiphum euphorbiae* of different sizes were obtained from laboratory cultures maintained on potato plants. Large aphids were produced on the inflorescences, and very small aphids were produced on the mature leaves of old plants. The aphids were flown for short periods and put on pea (*Pisum sativum*) seedlings. The numbers of larvae produced each day are given in Table 2. The length of the forewing was taken as an index of the size of the aphids.

TABLE 2—NUMBERS OF LARVAE PRODUCED EACH DAY FOR THE FIRST THREE DAYS AFTER SETTLING DOWN BY ALATAE OF *Macrosiphum euphorbiae* OF DIFFERENT SIZES

Wing length	First day	Second day	Third day
60	6	4	2
60	4	5	2
57	2	1	5
57	3	3	2
55	0	2	1
55	0	3	3
55	0	2	3
52	0	2	2
50	0	1	1
50	0	2	1
50	1	2	1
50	2	2	0
47	0	2	0
46	0	1	3
45	0	0	0
45	1	0	0
45	0	0	0
43	0	0	0

The largest aphids produced several larvae during the first 24 hr and additional larvae by the next day when their abdomens were fully distended and they had lost the ability to fly. Intermediate sized aphids did not begin to bear larvae until the second day, while very small aphids did not produce any larvae at all until the fourth or fifth day of the experiment, by which time their flight muscles had undergone considerable degeneration.

A number of young alatae of *M. euphorbiae* of various sizes were dissected and their ovaries examined. Although in the smaller aphids there were frequently fewer ovarioles and fewer embryos in each ovariole, the principal effect of reduced size was the less advanced state of development of the embryos. The largest aphids contained several almost fully developed embryos when embryo development was arrested, whereas in small aphids the largest embryos were not fully developed and required a few days' growth before they were ready to be born.

ABILITY TO UNDERTAKE LONG FLIGHTS ON SUCCESSIVE DAYS

As the flight muscles of alatae of *A. fabae* were apparently unaffected until the aphids had been settled down feeding and reproducing for a few days, it might be expected that the aphids would retain the ability to undertake long flights until they lost the ability to fly altogether. The following experiment was designed to test this hypothesis.

A number of aphids were flown attached to pins. The flight of aphids *A* and *B* was interrupted after 8 hr on the first day: on the second and third days they were flown to exhaustion; the remainder of the aphids were only flown to exhaustion on the third day. Between flights they were kept on bean leaves.

All the aphids were able to fly on three consecutive days. On the second and third days they were more reluctant to fly, but once started they flew well. None

TABLE 3—INDIVIDUAL REPRODUCTIVE AND FLIGHT HISTORIES OF A NUMBER OF ALATAE OF *A. fabae*

Aphid	First day		Second day		Third day		Fourth day	Total larvae	Total flight
	Flight	Larvae	Flight	Larvae	Flight	Larvae	Flight		
	(hr)		(hr)		(hr)		(hr)		(hr)
A	8	11	4½	3	1½	0	0	14	14
B	8	10	4½	1	1½	0	0	11	13
C	3	12	3	4	1½	5	0	21	7½
D	3	9	3	2	½	3	0	14	6
E	3	9	3	7	1½	2	0	18	7½
F	3	9	3	6	1½	4	0	19	7½
G	3	9	3	4	2	5	0	18	8

of them could be induced to fly at all on the fourth day. It appears therefore that the ability of alatae of *A. fabae* to undertake long flights can be retained for at least 2 days after the aphids settle down.

DELAYED ONSET OF FLIGHT MUSCLE HISTOLYSIS

When aphids were kept off their host plant, or when they were kept on a host plant from the final ecdysis in darkness and without having engaged in any locomotor activity, so that they did not actually settle down to feed and reproduce, muscle histolysis was delayed. The aphids retained the ability to fly for as long as they could be prevented from settling down. The flight muscles of alatae of *A. fabae* which were kept at 13°C in a Petri dish for 5 days, and did not reproduce at all, remained intact, whereas in aphids which were allowed to settle down on host plants they began to degenerate within 3 days.

Not only was it possible to delay muscle histolysis in this way before any larvae at all had been born, but it was also possible to delay it after the birth of the first batch, and even after the birth of the additional larvae that complete their embryonic development subsequent to the birth of the first batch. This is shown in the following experiment.

Alatae of *A. fabae* were flown for 1 hr on pins and put on mature broadbean leaves in a dark cupboard. Overnight they gave birth to an average of twelve larvae. The alatae were then put on a single bean leaf and returned to the cupboard where they were left for a further 3 days. When they were examined they had produced an additional thirty-two larvae (average of four larvae each) and when put in the light they all took off. They were then attached to pins and flown for a further 1 hr and returned to the leaf. During the next 24 hr they produced an average of four to six larvae each, and after this they could no longer be induced to

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fly. The results of the experiment are summarized in Table 4. The aphids had given birth to the larvae of the first batch, a few further larvae had been born, and histolysis of the flight muscles was then delayed for 2 days.

TABLE 4—DELAYED ONSET OF MUSCLE BREAKDOWN IN ALATAE OF *A. fabae* AFTER THE BIRTH OF A NUMBER OF LARVAE

	Day	1	2	3	4	5	6
Delayed muscle breakdown	Flight ability	*F*	*F*	—	—	*F*	*M*
	no. of larvae	12		4·0		4·6	
Normal muscle breakdown (from Table 1)	Flight ability	*F*	*F*	*F*	*M*	—	—
	no. of larvae	10	4	3	—	—	

F — Aphids flew when stimulated. *M* — Aphids could not be stimulated to fly.

Dissections of alatae of *A. fabae* showed that when embryonic development was arrested they contained about twelve (range 8–15) fully formed embryos, and several slightly less developed ones which had less pigment in the body and eyes than fully formed embryos and were only about three-quarters of their size. The fully formed embryos and these less developed ones were born before the flight muscles degenerated. As the muscles degenerated, development of the numerous smaller embryos in the ovarioles was resumed.

DISCUSSION

The species of aphids used in this study belong to three taxonomic groups according to BAKER's (1920) classification. *S. mali*, *A. fabae*, and *B. brassicae* belong to the sub-tribe Aphidina; *M. persicae*, *M. euphorbiae*, and *M. viciae* to the sub-tribe Macrosiphina; and *T. saligna* and *C. pinicola* to the tribe Lachnini. It is interesting that there were differences in the characteristics of the three groups. In Aphidina the young alatae contained a large number of fully formed embryos that were born before the aphids lost the ability to fly; in Lachnini only rudimentary embryos were present in the young alatae and these were not born until well after the aphids had become incapable of flight. The three species of Macrosiphina tended to lie between these two groups in their characteristics and to form a graded series between them: *M. persicae* resembled Aphidina but contained fewer fully formed embryos, *M. euphorbiae* had less developed embryos, and *M. viciae* tended to behave even more like the Lachnini in that larviposition did not normally occur until the aphids had been settled down for several days and the flight muscles had undergone considerable degeneration. It is also significant that breakdown of the flight muscles of the Aphidini and of *M. persicae* began later than in the other species.

HAINE (1955) could find no evidence that the flight muscles of some species of tree aphids of the tribe Callipterini degenerated, and in view of the active mode of life of the species she studied it is probable that their muscles do not break down and they retain the ability to fly for their whole lives.

In each of the species that was studied here embryo development was arrested soon after metamorphosis and it was resumed after the aphids had settled down to feed. Aphids which contained a number of fully formed embryos also had a few embryos which were nearly fully formed but were not fully pigmented or as large as they normally are when they are born. Before the aphids lost the ability to fly all the fully formed embryos and these few additional ones were born. Further embryo development did not occur without the flight muscles degenerating, and the two processes appeared to be related. It is unlikely that the developing embryos of aphids depend in any way on materials from the degenerating muscles as ovary development apparently does in ants, termites, and *Aëdes communis*, because when the aphids settle down they begin to feed, generally on young or senescing plant tissue that is rich in nitrogen. In this respect aphids resemble more the parasitic flies *Carnus* and *Lipoptena* in which the flight muscles degenerate after the flies have become attached to their host and have begun to feed on it (MERCIER, 1928). The breakdown products of the muscles of aphids would probably become available for embryo development, but embryo development would not be dependent on them, and if there is a relationship between the two processes it could be that they are controlled by a common factor which is brought to bear after the aphids have been settled down on a plant for 1 or 2 days. The fact that larvae of *M. euphorbiae* and *M. viciae* can be born prematurely and can live suggests that the embryo development that normally occurs after the aphids settle down and before their muscles degenerate may merely be due to an increase in size of the embryos due to water uptake. But this is not entirely the case in *A. fabae* where the young alatae contain a maximum of fifteen embryos with fully pigmented bodies and eyes, and yet up to twenty-one larvae can be born before the muscles degenerate.

Most of the aphids in the experiments lost the ability to fly within 2–3 days of settling down on a host plant. It has been shown by JOHNSON (1957) that aphids of some species can remain for several days on plants without settling down to feed and reproduce and without losing the ability to fly. In this way the length of time that they can retain the ability to fly can be considerably extended. But the reproductive capacity of alatae before they lose the ability to fly is limited and therefore the number of plants the aphids can reproduce on must be restricted; it will be more restricted in some species than in others and within a species it will be more restricted in the smaller individuals.

BROADBENT (1949) showed that the numbers of flights attempted by alatae of *M. persicae* and *B. brassicae* in a glass chamber decreased with increasing age, and that aphids 4–5 days old made no attempted flights at all. As the aphids were confined between flight tests on host plants and reproduced on them, it is likely that the decrease in flight activity was related to the breakdown of the flight

muscles. The fact that some aphids retained the ability to fly for longer than 3 days suggests that they had spent some time either on the cages covering the plants or "sitting" but not settled down on the plants.

While the loss of the ability to fly appears to be due to the degeneration of the flight muscles, there is concurrently a reduction in the activity of the aphids. Before the flight muscles have begun to degenerate alate aphids can readily be disturbed and they may then remain unresponsive to host plants for some time; after the muscles have begun to degenerate the aphids are less readily disturbed, but if they are caused to withdraw their stylets they settle down again much more readily than aphids which still have their flight muscles intact. After they have lost the ability to fly alate aphids commonly remain settled down feeding and reproducing for the rest of their lives unless they are provoked by failing food-supply or disturbances to withdraw their stylets and move about.

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DIE STEUERUNG DER OVARFUNKTION BEI DER OVOVIVIPAREN SCHABE *LEUCOPHAEA MADERAE* (FABR.)*

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Abstract—In the ovoviviparous roach *Leucophaea* the ovaries are inactive during pregnancy. The corpora allata show histologically an activity cycle (Fig. 3) which is correlated to the ovary cycle. During pregnancy the corpora allata are inactive. Implantation of active c. allata from nymphs into pregnant females results in a renewed ovarial activity. The eggs ripen, i.e. they grow, and yolk is deposited. This confirms the findings of DAY (1943), JOLY (1945), SCHARRER (1943, 1946), THOMSEN (1940), WIGGLESWORTH (1936), and others that the c. allata produce a gonadotropic hormone in the adult female of many insects which is responsible for the ripening of the eggs in the ovary.

The c. allata, and through them the ovary, can be activated during pregnancy by cutting the c. allatum nerve, or the median c. cardiacum nerve, or by destroying a certain part of the protocerebrum. This operation was also effective when the neurosecretory cells and the pathways of the neurosecretory material were untouched. From this it can be concluded that a nervous centre in the brain is responsible for an inhibition of the c. allata during pregnancy. But activation of the c. allata after these operations only occurs when the nerve connecting the c. allata with the suboesophageal ganglion remains intact. Thus the activity of the c. allata seems to be controlled by inhibitory centres in the brain and by stimulatory centres in the suboesophageal ganglion.

The same inhibitory mechanism is also effective in the last nymphal stage when metamorphosis is initiated by a temporary inactivity of the c. allata.

The c. allata can be activated during pregnancy by removal of the oötheca from the brood sac. This activation does not take place, however, if developing eggs are implanted into the body cavity immediately after the removal of the oötheca. This indicates that the developing eggs in the brood sac release a substance which causes the brain to inhibit the c. allata. It is probable that the yolk system is responsible for this effect.

EINLEITUNG

DURCH DAY (1943), JOLY (1945), SCHARRER (1946), THOMSEN (1940), WIGGLESWORTH (1936) und andere ist nachgewiesen worden, dass die Corpora allata vieler Insekten im adulten Weibchen ein gonadotropes Hormon abgeben,

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Teil einer Dissertation (Anregung und Leitung: Prof. Dr. M. LÜSCHER). Herrn Prof. F. E. LEHMANN (Bern) und Frau Dr. B. SCHARRER (New York) danke ich für die Durchsicht des Manuskriptes und für viele wertvolle Anregungen. Zu grossem Dank fühle ich mich meiner Frau verpflichtet, die einen Grossteil der histologischen Präparate anfertigte und bei deren Auswertung mithalf.

ohne das im Ovar die Eireifung unter Dottereinlagerung nicht möglich ist. Die meisten dieser Autoren haben auch festgestellt, dass nach Kastration die C. allata vergrössert werden. Sie haben deshalb vermutet, dass ein Keimdrüsenhormon auf die C. allata zurückwirkt und diese hemmt.

Für eine Analyse dieser Wirkung des Ovars auf die C. allata schien *Leucophaea* besonders geeignet, da bei dieser ovoviviparen Schabe die Ovarien während der Trächtigkeit vollkommen inaktiv sind; die Eier wachsen nur ganz wenig und es wird kein Dotter eingelagert. Durch experimentelle Eingriffe während dieser Periode lassen sich die Faktoren analysieren, welche zu einer Aktivierung der C. allata und des Ovars führen. So ergibt sich die Möglichkeit, sowohl die Steuerung der C. allata als auch diejenige der Ovarfunktion abzuklären.

Wie in einer vorläufigen Mitteilung (LÜSCHER und ENGELMANN, 1955) schon nachgewiesen wurde, wirkt der Eikokon stofflich auf das Gehirn, das seinerseits die C. allata hemmt. Diese produzieren kein, oder nur in ungenügender Menge, gonadotropes Hormon und die Eireifung unter Dottereinlagerung bleibt deshalb aus.

In der vorliegenden Arbeit werden zunächst die Beziehungen zwischen den C. allata und den Ovarien durch histologische Untersuchungen aufgezeigt. Es folgt eine experimentelle Analyse der drei an der Steuerung der Ovarfunktion beteiligeten Mechanismen (gonadotrope Wirkung der C. allata; Steuerung der C. allata vom Gehirn aus; Wirkung des Eipaketes im Uterus auf Gehirn und C. allata).

METHODEN UND TECHNIK

Die Versuchstiere wurden bei 26°C und bei 75–80% rel. Luftfeuchtigkeit gehalten. Gefüttert wurde mit Hundekuchen und Karotten (ENGELMANN, 1957).

Zur Durchführung der Experimente wurden die Schaben mit CO_2 narkotisiert. Die Elektrokoagulation von Gehirnpartien erfolgte mit dem "Dermo-Pytor" der Firma Sanitas, Berlin.

Die C. allata wurden zusammen mit den C. cardiaca und den Gehirn fast ausnahmslos in Bouin-Duboscq fixiert. Für den Nachweis von Neurosekret fand die Methode von GOMORI Anwendung (GOMORI, 1941), doch wurde teilweise auch mit Paraldehyd-Fuchsin (HALMI, 1952) gefärbt. Für die Verfolgung feiner Nervenfasern im Gehirn brachte eine Imprägnierung der Gewebe mit Silbernitrat in Verbindung mit Harnstoff (UNGEWITTER, 1951) die besten Ergebnisse. Die Grössenmessung der Eier in den Ovarien wurde an fixiertem Material nach Anfärbung durch Boraxkarmin mit einem Messokular vorgenommen. In den Tabellen ist jeweils der Durchschnitt aus vier Eiern eingetragen.

Die Volumina der C. allata wurden wie folgt berechnet. Nach Herstellung der Schnittserien wurde jeder Schnitt mit Hilfe des Abbe'schen Zeichenapparates gezeichnet und planimetriert. Aus der gemessenen Fläche und der Schnittdicke ergab sich das Volumen. Da die Drüsen immer die gleiche Behandlung erfuhren (Fixieren, Einbetten usw.), sind alle Werte vergleichbar. Sie dürften etwas zu klein sein, da die durch das Fixieren bedingte Schrumpfung nicht berücksichtigt

wurde. In den Tabellen und in Abb. 3 bedeutet jeder Wert das durchschnittliche Volumen eines C. allatum.

Die Bestimmung der Kerndichte der C. allata erforderte folgende Überlegung. Bei der Auszählung der Kerne werden ausser den ganz im Schnitt enthaltenen auch alle angeschnittenen Kerne, die als Kalotten von beiden Seiten in den Schnitt hineinragen gezählt, also alle Kerne der Nachbarschnitte, deren Kernmittelpunkt nicht weiter als r (r = Kernradius) von der Schnittoberfläche entfernt sind. Bei der Auszählung der Fläche F werden alle Kerne erfasst, die in dem Volumen $[F \times (D+2r)]$ liegen (D = Schnittdicke). Für die Anzahl der Kerne, die sich in der Volumeneinheit (V = 1 mm³) befinden, ergibt sich folgende Formel:

$$K = \frac{Z \times V}{F \times (D+2r)}$$

(K = Kernanzahl pro Volumeneinheit; Z = gezählte Kerne pro Schnittfläche F). Es muss hier aber berücksichtigt werden, dass bei grosser Kerndichte u.U. zwei Kernkalotten übereinander liegen können, sodass nur ein Kern gezählt wird. Bei sehr dichter Kernpackung werden deshalb zu wenig Kerne berechnet. In unserm Falle stimmen jedoch die errechneten Kernzahlen im allgemeinen gut mit den Zahlen überein, die man aus dem Flächenanteil der Kerne an einer Schnittoberfläche errechnet.

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DER ZYKLUS DER OVAR-AKTIVITAT

Das Wachstum der grössten Oocyten in den Ovarien ist in Abb. 3 dargestellt. Bei der Metamorphose sind sie ca. 0,91 mm lang (Abb. 4). Bald nach der Metamorphose beginnen sie zu wachsen, wobei immer Dotter eingelagert wird. Ca. 33 ± 4 Tage nach der Adulthäutung, wenn die Eier eine Länge von 4,4 mm erreicht haben (Abb. 1, 3) erfolgt die erste Ovulation. Während dieser Aktivität der Ovarien sind die Anhangsdrüsen des Geschlechtsapparates ebenfalls aktiv. Vor allem werden in der Drüse A_2 (ENGELMANN, 1957) sehr grosse Mengen von Sekret produziert. Bei der Ovulation wird alles Sekret aus den Anhangsdrüsen ausgeschüttet und zur Kokonbildung verwendet.

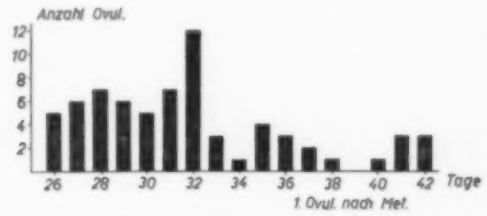

ABB. 1. Dauer der Eireifung nach der Metamorphose.

Während der ersten 15 Tage der folgenden Trächtigkeit wachsen die nächsten Oocyten sehr wenig (Abb. 3). Vom 15. bis zum 60. Tage der Trächtigkeit wird

jedoch kein weiteres Wachstum der Oocyten beobachtet. Sie sind während dieser Zeit etwa 0,70 mm lang. In den letzten Tagen der Trächtigkeit erfolgt wieder ein geringes Wachstum, sodass zur Zeit der Geburt der Jungen die Eilänge 0,9–1,0 mm misst. Dies entspricht etwa der Eilänge im Zeitpunkt der Metamorphose. Wie vor der Metamorphose wird auch bei dem Wachstum vor einer Geburt nie Dotter in den Oocyten eingelagert. Die Anhangsdrüsen des Geschlechtsapparates bleiben während der ganzen Trächtigkeit inaktiv und nur gegen Ende der Trächtigkeit kann histologisch eine schwache Sekretproduktion nachgewiesen werden.

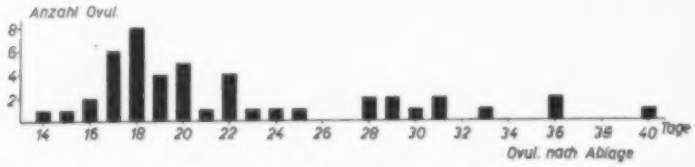

ABB. 2. Dauer der Eireifung nach einer normalen Ablage des Eipaketes.

Nach der Ablage der Jungen beginnt ein rasches Wachstum der Eier in den Ovariolen. In 22±6,3 Tagen reifen die Eier zur Ovulationsgrösse (Abb. 2). Wiederum werden synchron mit den Ovarien die Anhangsdrüsen aktiv, und man beobachtet am Ende dieser Aktivitätsperiode in den Drüsenschläuchen etwa gleich viel Sekret wie am Ende der ersten Eireifungsperiode, obwohl die Sekretionsperiode wesentlich kürzer ist. Die Drüsenzellen müssen demnach in der zweiten oder folgenden Eireifungsperiode aktiver sein als während der ersten Eireifungsperiode nach der Metamorphose.

Ovarien und Anhangsdrüsen zeigen also gleichzeitig eine zyklische Aktivität, die offenbar primär von der Anwesenheit von Eiern im Uterus abhängig ist.

DER ZYKLUS DER CORPORA ALLATA-AKTIVITÄT

Um die Beziehung zwischen Eireifung und C. allata—Aktivität zu erfassen, wurden die Volumina und Kerndichten der C. allata während eines Zeitraumes von 135 Tagen nach der Metamorphose bestimmt. Diese Zeit umfasst die erste Eireifungsperiode, die erste Trächtigkeitsperiode und die zweite Eireifungsperiode. Als Mass für die Aktivität der C. allata kann man wohl das Volumen dieser Drüse betrachten (LUKOSCHUS, 1956; LÜSCHER und ENGELMANN, 1955; MENDES, 1948; NAYAR, 1953, 1955, 1956; PALM, 1947; PFLUGFELDER, 1938; WIGGLESWORTH, 1936). Da sich die Kernanzahl in der Drüse beim adulten Weibchen von *Leucophaea* nicht verändert (Mitosen wurden nie beobachtet), dürfte auch die Kerndichte ein Mass für die Aktivität sein; sie ist umgekehrt proportional der Aktivität. Die Bestimmung der Kerndichte erfordert weniger Zeit als die Berechnung des Drüsenvolumens. Die Bestimmung hat jedoch nur einen Sinn, wenn, wie in vorliegendem Falle, die Kernzahl und die Kerngrösse unverändert bleiben. Das richtigste Mass für die Aktivität wäre wohl die Gesamtmenge des Cytoplasmas.

Die Volumina der C. allata nehmen nach der Metamorphose rasch zu und erreichen um den 20. Tag einen Höhepunkt (LÜSCHER und ENGELMANN, 1955)*

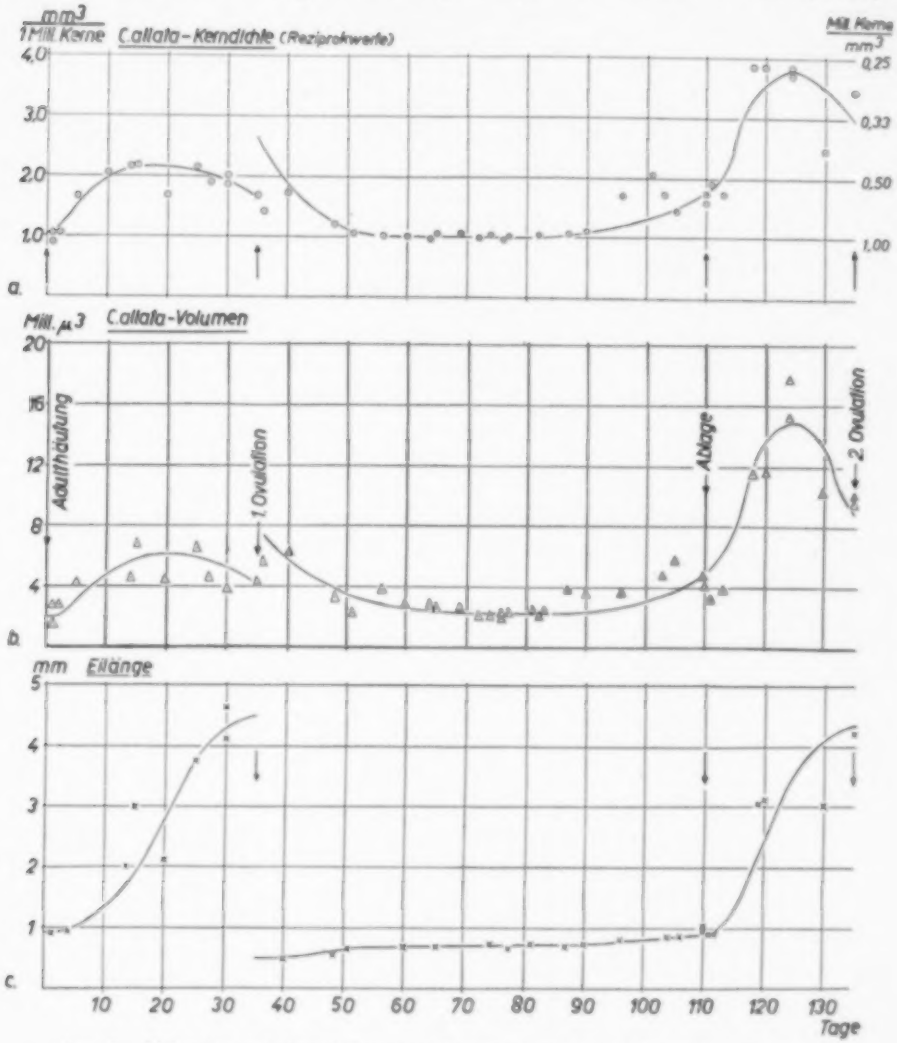

ABB. 3. Volumina und Kerndichten der Corpora allata, sowie Längen der grössten Eier in den Ovarien während 135 Tagen nach der Metamorphose. Jeder Punkt in den Kurven *a* und *b* entspricht dem durchschnittlichen Volumen bzw. der Kerndichte eines C. allatum eines Tieres. Die Unterbrechung in den Kurven *a* und *b* im Zeitpunkt der ersten Ovulation erklärt sich dadurch, dass für die Trächtigkeits- und für die nachfolgende Eireifungsperiode auch ältere Weibchen mitberücksichtigt wurden.

* Die in dieser Mitteilung gegebenen Werte sind infolge eines Berechnungsfehlers 140 mal grösser als die hier publizierten richtigen Werte.

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(Abb. 3). Bis zur ersten Ovulation (etwa 30–35 Tage nach der Metamorphose) wird das Volumen der C. allata wieder ein wenig reduziert. Umgekehrt verhält sich die Kernzahl pro 1 mm³. Bei der Metamorphose sind in 1 mm³ Drüsenvolumen etwa 1,0 Millionen Kerne enthalten; die Kerne machen etwas weniger als $\frac{1}{4}$ des gesamten Drüsenvolumens aus. Wenn das Volumen der C. allata während der ersten Eireifungsperiode das Maximum erreicht hat, kommen auf 1 mm³ nur noch 0,46 Millionen Kerne; nur $\frac{1}{10}$ des Drüsenvolumens besteht aus Kernen (Abb. 4).

Nach der ersten Ovulation, d.h. während der Trächtigkeit, nimmt das Volumen der C. allata rasch ab, während die Kerndichte entsprechend zunimmt. Es werden die gleichen Drüsenvolumina und Kerndichten berechnet wie zur Zeit der Metamorphose (Abb. 3, 4). Zwischen dem 15. und 55. Trächtigkeitstag bleibt die Kerndichte sodann konstant, etwa 1,0 Millionen pro mm³. Gegen Ende der Trächtigkeit ist jedoch wieder eine geringe Volumenzunahme und entsprechend eine Kerndichteabnahme in den C. allata festzustellen.

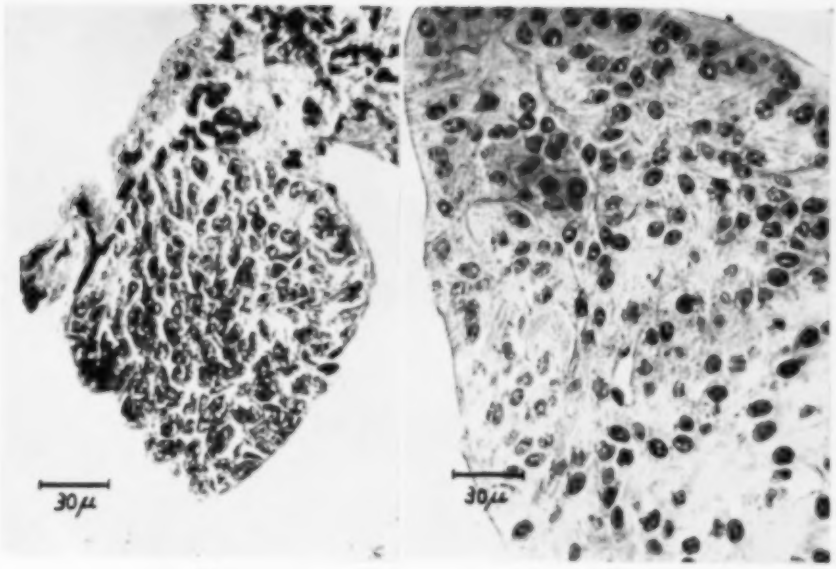
Sofort nach der Geburt der Jungen steigt das Volumen der C. allata rasch an; nach etwa 14 Tagen ist es mehr als doppelt so gross als das Maximum während der ersten Eireifungsperiode (Abb. 3). Entsprechend dieser grossen C. allata beträgt die Kerndichte nur noch 0,26 Millionen/mm³; nur—$\frac{1}{17}$ des Drüsenvolumens besteht aus Kernen. Auch in dieser zweiten Eireifungsperiode erfolgt kurz vor der Ovulation eine Volumenverminderung und damit eine Erhöhung der Kerndichte.

Da während des ganzen Zyklus keine Mitosen in den C. allata beobachtet werden konnten, und da der Kernradius immer etwa 3,8 μ misst, das Volumen der Kerne sich also nicht verändert, werden die Volumenschwankungen der C. allata wohl ausschliesslich durch Veränderungen des Cytoplasmagehaltes verursacht. Die Volumina der C. allata schwanken jedoch individuell ziemlich stark. Für die Kerndichte ist die Streuung kleiner als für die Volumina (Abb. 3); sie gibt deshalb wohl ein besseres Mass für den physiologischen Zustand der C. allata.

EXPERIMENTELLE ANALYSE DER STEUERUNG DES OVARIALZYKLUS

Für alle im Folgenden geschilderten zur Abklärung der Steuerung des Ovarialzyklus durchgeführten Experimente wurden trächtige Weibchen verwendet, deren C. allata nach den histologischen Untersuchungen vollkommen inaktiv sind. Die Ergebnisse der Versuche sind in den Tabellen 1–5 dargestellt. Für die C. allata werden als Indizien der Aktivität in den Tabellen das durchschnittliche Volumen einer Drüse und die Zahl der Kerne pro mm³ Drüsenvolumen angegeben. Die physiologische Aktivität geht aus der Länge der grössten Oocyten (Eilänge) in den Ovarien hervor.

Als Kontrollen können die in Tabelle 1 zusammengestellten Serien betrachtet werden. Eine Serie von normalen trächtigen Weibchen zeigt die völlige Inaktivität (Tabelle 1a). Entfernt man den Eikokon zu irgend einem Zeitpunkt der Trächtigkeit, so werden die C. allata nach kurzer Zeit aktiviert (Tabelle 1b). Diese Aktivierung nach Entfernung des Kokons darf als Mass für den maximal



(a) (b)

ABB. 4. Schnitte durch die Corpora allata von *Leucophaea maderae*. (a) Inaktives Corpus allatum. Es ist wenig Cytoplasma vorhanden; die Kerndichte ist gross. (b) Aktives Corpus allatum (Teil eines Schnittes). Sehr viel Cytoplasma; Kerndichte klein.

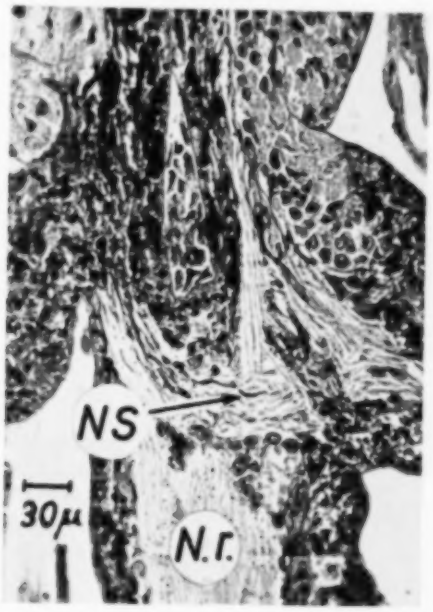


ABB. 6. Corpora cardiaca, caudales Drittel. In den sich überkreuzenden Nerven ist Neurosekret nachweisbar. *N.S.* = Neurosekret. *N.r.* = Nervus recurrens.

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möglichen Effekt nach einem operativen Eingriff gelten. Der Operationseffekt liegt meist unter dem möglichen Effekt. Der Grund hierfür liegt wohl in einer allgemeinen Störung des Tieres durch die Operation. Die Nahrungsverweigerung in den ersten Tagen der Operation kann sich in Störungen der Tätigkeit der C.

TABELLE 1—KONTROLLVERSUCHE

	Operation Tage nach Ovulation	Fixierung		C. allatum		Ovar Eilängen (mm)
		Tage nach Operation	Tage nach Ovulation	Volumen (Millionen μ^3)	Kernanzahl pro 1 mm^3 (Millionen)	
a. Normale Tiere während der Trächtigkeit	—	—	25	2,68	0,98	0,66
	—	—	29	2,77	1,01	0,66
	—	—	30	2,60	0,97	0,68
	—	—	34	2,40	0,97	0,68
	—	—	37	1,74	1,01	0,66
	—	—	39	2,03	0,96	0,67
	—	—	41	2,41	1,01	0,66
	—	—	41	1,85	1,01	0,65
	—	—	42	2,35	0,98	0,65
	—	—	47	2,03	0,97	0,69
	—	—	52	4,00	0,94	0,68
	—	—	55	4,03	0,92	0,70
	—	—	61	3,88	0,60	0,89
Total 13 Tiere	—	—	25–61	2,66 ± 0,76	0,94 ± 0,11	0,69 ± 0,06*
b. Nach Entfernen des Eipaketes	24	17	41	11,05	0,33	1,05
	26	15	41	6,02	0,39	3,30
	36	15	51	9,15	0,30	1,15
	34	15	49	10,60	0,41	2,81
	30	15	45	10,80	0,41	2,05
Total 5 Tiere	24–30	15–17	41–51	9,50 ± 2,12	0,36 ± 0,01	2,07 ± 0,99*
c. Nach Entfernen des Eipaketes und anschliessendem Hungern	32	13	45	4,95	0,60	1,07
	34	13	47	3,58	0,84	0,83
	35	13	48	5,13	0,55	0,90
	31	12	43	4,32	0,70	0,76
	36	14	50	5,90	0,73	0,83
	30	14	46	3,53	0,74	0,74
Total 6 Tiere	30–36	12–14	43–50	4,56 ± 0,98	0,70 ± 0,11	0,86 ± 0,24*

* ± bedeutet hier und in allen folgenden Tabellen die mittlere quadratische Abweichung (standard deviation).

allata äussern. Nach Entfernen des Kokons und anschliessendem Hungern (Tabelle 1c) wird bei weitem nicht eine Aktivität der C. allata erreicht, die nach normaler Entnahme des Kokons beobachtet werden kann. Der Einfluss des Hungers kann so stark sein, dass die C. allata fast völlig inaktiv bleiben.

Auf Grund der Experimente lassen folgende Werte für die C. allata auf Inaktivität bzw. Aktivität schliessen:

Volumen einer Drüse	Kernzahl pro mm³	
<4 Millionen μ^3	>0,77 Millionen	Inaktivität
4–6 Millionen μ^3	0,63–0,77 Millionen	schwache Aktivität
6–9 Millionen μ^3	0,42–0,63 Millionen	mittlere Aktivität
>9 Millionen μ^3	<0,42 Millionen	starke Aktivität

In den Ovarien lässt eine Eilänge (Oocyte) von mehr als 0,85 mm auf eine physiologische C. allata-Aktivität schliessen.

Die Wirkung der Corpora allata auf die Eireifung

Werden bei *Leucophaea* die C. allata extirpiert, so wird in den Ovarien kein Dotter in die Oocyten eingelagert und die Anhangsdrüsen des Geschlechtsapparates produzieren kein Sekret (SCHARRER, 1946). Bei Implantation von C. allata aus normal gefütterten in Hungertiere (JOHANSSON, 1955) reifen in diesen die Eier. Körperfremde C. allata können demnach in Tieren mit inaktiven C. allata (Hungertiere haben immer inaktive C. allata) eine Eireifung in den Ovarien bewirken. Aber auch bei Implantation von larvalen aktiven C. allata in trächtige Weibchen reifen die Eier in den Ovarien der Wirtstiere (Tabelle 3). In diesem Zusammenhang stellt sich die Frage, ob die Ovarien und Anhangsdrüsen unabhängig von einander durch die C. allata aktiviert werden. Da bei Kastration die Anhangsdrüsen aktiv bleiben (SCHARRER, 1946) und da auch nach Extirpation der Anhangsdrüsen in den Ovarien Eier reifen (ENGELMANN, 1957) ist die Wirkung der C. allata auf Ovarien und Anhangsdrüsen sicher unabhängig.

Durch Implantation und Extirpation wurde nicht nur bei *Leucophaea* eine Beziehung zwischen C. allata und Ovarien experimentell nachgewiesen (ENGELMANN und LÜSCHER, 1956; LÜSCHER und ENGELMANN, 1955; SCHARRER, 1946); auch bei vielen anderen Insekten besteht diese Abhängigkeit (BODENSTEIN, 1947; CLEMENTS, 1956; DETINOVA, 1945; JOLY, 1945, 1950; PALM, 1950; THOMSEN, 1940, 1942, 1948a; VOGT, 1940a, b; WIGGLESWORTH, 1936). Die nach den histologischen Untersuchungen zu erwartende Korrelation zwischen Ovarien und C. allata ist also experimentell erwiesen.

Die Steuerung der Corpora allata vom Gehirn aus

Die Durchtrennung der C. allata-Nerven zeigt, dass die C. allata vom Gehirn abhängig sind (LÜSCHER und ENGELMANN, 1955; SCHARRER, 1952a). Die Steuerung der C. allata-Funktion kann vom Gehirn aus entweder nervös oder durch das Neurosekret erfolgen. Zur Abklärung dieser Frage wurden bei trächtigen Weibchen die verschiedenen Nervenverbindungen durchtrennt oder Gehirnpartien durch Elektrokoagulation ausgeschaltet (ENGELMANN und LÜSCHER, 1957).

Die Innervierung der C. allata ist in Abb. 5 dargestellt. Wie bei den meisten Insekten werden die Corpora cardiaca durch ein medianes und ein laterales Nervenpaar (Nervus corporis cardiaci interior und exterior nach CAZAL (1948); N.c.c. I and II nach HANSTRÖM (1940)) innerviert, Beide vereinigen sich

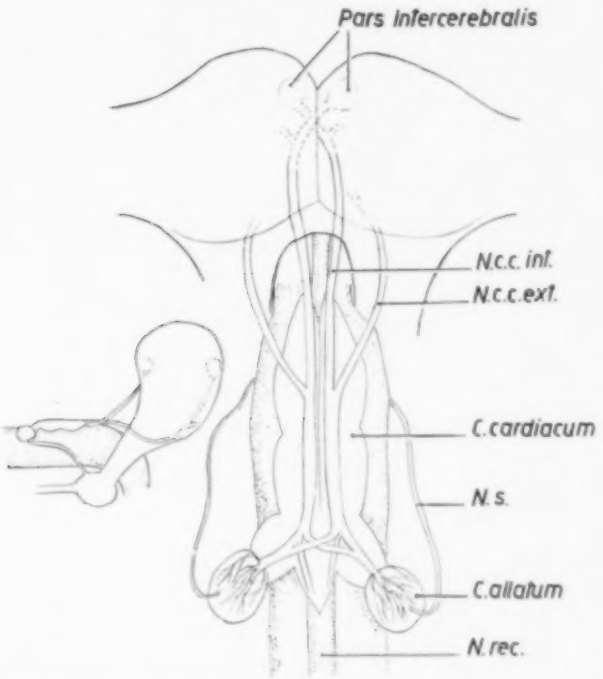

ABB. 5. Innervation der Corpora cardiaca und der Corpora allata vom Protocerebrum und Suboesophagialganglion aus (halbschematisch). *N.c.c. int.* = Nervus corporis cardiaci interior (medianer Cardiacum-Nerv). *N.c.c. ext.* = Nervus corporis cardiaci exterior (lateraler Cardiacum-Nerv). *N.s.* = Nerv der zum Suboesophagialganglion führt. *N. rec.* = Nervus recurrens.

jederseits zu einem einheitlichen Strang, der sich im letzten Drittel des Corpus cardiacum in drei Aeste aufspaltet. Ein Ast vereinigt sich mit dem Nervus recurrens. Die beiden andern vereinigen sich mit den entsprechenden Ästen der andern Seite und innervieren die C. allata. Jedes Corpus allatam steht ausserdem durch einen weiteren Nerv, der auch für *Gryllus* beschrieben wurde (HUBER, 1955), mit dem Suboesophagialganglion in Verbindung.

Die Durchtrennung der Nervenverbindung zwischen Protocerebrum und C. allata in den caudalen Seitenästen der C. cardiaca (LÜSCHER und ENGELMANN, 1955) macht eine direkte Beeinflussung der C. allata durch Neurosekret unmöglich. Nach dieser Nervendurchtrennung nehmen die C. allata an Volumen zu, sie werden aktiviert. Wenn eine Neurosekretwirkung bestehen würde, so müsste

demnach das Fehlen von Neurosekret zu einer Aktivierung der C. allata führen. Bei Durchtrennung des medianen Cardiacum-Nervs kann jedoch der gleiche Effekt erzielt werden (SCHARRER, 1952a), obschon in diesem Fall Neurosekret von der intakten Seite zum C. allatum gelangen kann (Abb. 6). Damit ist erwiesen, dass das Neurosekret nicht direkt an der C. allata-Steuerung beteiligt ist. Zudem lässt sich histologisch bei *Leucophaea* keine Beziehung zwischen der vorhandenen Neurosekretmenge und der C. allata-Aktivität finden. Neurosekret ist in allen Aktivitätsphasen der C. allata in mehr oder weniger grossen Mengen vorhanden. Dieser Befund gibt eine weiter Bestätigung dafür, dass Neurosekret die C. allata nicht direkt steuert. Andererseits scheint aber das Vorhandensein von Neurosekret notwendig zu sein, damit eine Aktivierung der C. allata überhaupt zustande kommt, denn bei Fehlen von Neurosekret—dies kommt bei älteren trächtigen wie nicht trächtigen Weibchen manchmal vor—schrumpfen die Kerne der C. allata stark, was vielleicht auf eine Degeneration der Drüsen schliessen lässt. Bei diesen Weibchen reifen keine Eier in den Ovarien, da die C. allata inaktiv bleiben.

Um über die Bedeutung der verschiedenen Nerven und des Gehirns für die Steuerung der C. allata Aufschluss zu erhalten, wurden Nervdurchtrennungs—und Ausschaltversuche am Gehirn durch Elektrokoagulation durchgeführt.

Wenn die C. allatum-Nerven in den caudalen Seitenästen der C. cardiaca durchschnitten werden, so nimmt das Volumen der C. allata nach einigen Tagen zu, und die Kerndichte wird verringert (Tabelle 2a, b; vergl. Normalwerte Tabelle 1a), Wird dieser Nerv nur einseitig durchtrennt, so wird das C. allatum der durchtrennten Seite aktiviert (LÜSCHER und ENGELMANN, 1955). Mit der Durchtrennung wird also eine nervöse Hemmung aufgehoben. Entsprechend dieser C. allata-Aktivierung beginnen die Oocyten in den Ovarien zu wachsen; es wird Dotter eingelagert. Es stellt sich nun die Frage, welcher der beiden Cardiacum-Nerven die Steuerung vom Gehirn übermittelt. Es wurde daher versucht beide Nerven getrennt zu durchschneiden. Durch ein frontales Fenster in der Kopfkapsel konnten durch den Schlundring hindurch die Cardiacum-Nerven mit einer kleinen Pinzettenschere erreicht werden. Es gelang, das mediane Nervenpaar und in einigen Fällen auch den lateralen Nerv durchzutrennen. Eine getrennte Durchschneidung der beiden Cardiacumnervenpaare misslang. Eine Verletzung der Kaumuskulatur musste unter allen Umständen vermieden werden, da die C. allata bei Hunger ohnehin nur schwach aktiv werden können (Tabelle 1c). Die C. allata werden immer nach Durchtrennen der medianen Nerven aktiviert (Tabelle 2c, d). Das Mitdurchtrennen des lateralen Cardiacumnervs hatte keinen Einfluss. Er hat wohl für die Steuerung der C. allata keine Bedeutung.

Der mediane C. cardiacum-Nerv hat seinen Ursprung in der Pars intercerebralis des Gehirns. Um zu prüfen, ob die Pars intercerebralis für die Steuerung der C. allata verantwortlich ist, wurde sie durch Elektrokoagulation ausgeschaltet (Tabelle 2e). In keinem Falle wurden die C. allata aktiv; in den Ovarien reiften keine Eier. Da bei *Calliphora* (THOMSEN, 1948b, 1952) nach Ausschaltung der Pars intercerebralis überhaupt keine Eireifung mehr erfolgt, wurde sie in zwei Versuchen nur einseitig ausgeschaltet, sodass immer noch Neurosekret von der intakten Seite

TABELLE 2—VERSUCHE ZUR STEUERUNG DER CORPORA ALLATA FUNKTION

	Operation Tage nach Ovulation	Fixierung		C. allatum		Ovar Eilängen (mm)
		Tage nach Operation	Tage nach Ovulation	Volumen (Millionen μ^3)	Kernanzahl pro 1 mm^2 (Millionen)	
a. Durchtrennen der Allatum-Nerven (beidseitig)	52 33	10 16	62 49	6,69 8,20	0,36 0,37	1,39 1,14
Total 2 Tiere	33–52	10–16	49–62	7,45 ± 1,07	0,36 ± 0,00	1,26 ± 0,18
b. Durchtrennen eines Allatum-Nervs	34 32	10 10	44 42	5,05* 6,08*	0,65* 0,40*	0,85 0,88
Total 2 Tiere	32–34	10	42–44	5,56 ± 0,71	0,52 ± 0,17	0,87 ± 0,01
c. Durchtrennen oder Verletzen der medianen Cardiacum-Nerven (beidseitig)	29 37 32 27 31 37 35	12 14 13 12 13 13 12	41 51 45 39 44 50 47	5,53† 7,28† 15,10† 4,77 9,23 3,75 6,50	0,56† 0,46† 0,27† 0,51 0,45 0,65 0,57	1,10 1,91 1,87 0,85 1,08 0,89 0,89
Total 7 Tiere	27–37	12–14	39–51	7,38 ± 3,84	0,49 ± 0,03	1,23 ± 0,46
d. Durchtrennen eines medianen Cardiacum-Nerven	36 36	11 13	47 49	3,73‡ 6,87‡	0,70‡ 0,59‡	0,75 1,00
Total 2 Tiere	36	11–13	47–49	5,33 ± 2,69	0,65 ± 0,07	0,87 ± 0,18
e. Ausschalten der Pars intercerebralis	30 29 36§ 32§	13 13 14 12	43 42 50 44	2,76 2,60 2,76 1,56	0,96 0,97 0,87 0,89	0,80 0,80 0,75 0,84
Total 4 Tiere	29–36	12–14	42–50	2,42 ± 0,63	0,92 ± 0,02	0,79 ± 0,03
f. Vollständige Abtrennung der C. allata und Reimplantierung	36 33 34 34 29 30 31	13 13 13 13 14 14 14	49 46 47 47 43 44 45	2,97 3,15 3,85 4,16 —‖ —‖ —‖	0,72 0,77 0,53 0,63 0,71 0,66 0,68	0,80 0,73 0,79 0,69 0,70 0,73 0,79
Total 7 Tiere	29–36	13–14	43–49	3,53 ± 0,64	0,67 ± 0,08	0,75 ± 0,04

* Nur C. allatum der durchtrennten Seite berechnet.
† Nerven nur verletzt; in die C. cardiaca fliesst Neurosekret.
‡ Nur C. allatum der durchtrennten Seite berechnet.
§ Einseitig.
‖ Reimplantat ohne anhängende C. cardiaca.

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in den Insektenkörper gelangen konnte. Auch in diesen Fällen unterblieb eine Aktivierung der C. allata.

Die histologische Untersuchung der Nervenbahnen im Gehirn ergab dann, dass kurz vor dem Austritt der medianen Cardiacum-Nerven aus dem Gehirn eine dünne Bahn von lateral hinzustösst. Die Wurzel dieser Nervenbahn liegt wahrscheinlich im Protocerebrum in der Nähe der Protocerebralbrücke oder des Zentralkörpers. Dieses Zentrum ist wahrscheinlich für die Steuerung der C. allata-Funktion verantwortlich, da bereits eine schwache Verletzung dieser seitlichen Bahn die Hemmung der C. allata aufhebt. Eine Ausschaltung des Zentrums allein gelang nicht, da es vermutlich sehr dicht neben den Bahnen liegt, die aus der Pars intercerebralis kommen, so dass diese immer mitverletzt wurden. In drei Versuchen jedoch (Tabelle 2c) konnte in den Pars intercerebralis-Nerven Neurosekret nach der Operation nachgewiesen werden, während die C. allata deutlich enthemmt waren. Dies ist ein nochmaliger Hinweis dafür, dass die Zellen der Pars intercerebralis, die weiterhin Neurosekret produzieren konnten, die C. allata-Funktion nicht steuern.

Wurde im Gehirn einseitig ein Bezirk ausgeschaltet, der die neurosekretorischen Zellen einer Seite und ventralwärts noch einen grösseren Bereich umfasste, so wurde das C. allatum der gleichen Seite enthemmt. Demnach überkreuzen sich die steuernden Nerven im letzten Cardiacumdrittel nicht. Dieses Experiment liefert einen weiteren Beweis dafür, dass die neurosekretorischen Zellen die C. allata nicht steuern, da sonst wegen der Kreuzung der neurosekretorischen Bahnen im Gehirn das C. allatum der nicht gestörten Seite hätte enthemmt sein müssen.

Vom Protocerebrum werden die C. allata während der Trächtigkeit gehemmt. Man kann sich nun fragen, ob der Nerv, der das C. allatum mit dem Unterschlundganglion verbindet, eine Funktion im Zusammenhang mit der C. allata-Tätigkeit hat. Zur Prüfung dieser Frage wurden alle Nervenverbindungen zu den C. allata durchtrennt (Tabelle 2f). Die C. allata zeigten nur eine ganz geringe Aktivität. Dabei war es ohne Bedeutung, ob die Abtrennung der C. allata im letzten Cardiacumdrittel erfolgte oder ob die C. allata genau an der Grenzfläche zwischen C. allata und C. cardiaca abgetrennt wurden. Die nervöse Verbindung zum Unterschlundganglion ist also für das Zustandekommen einer Aktivität von Bedeutung. Es ist deshalb zu erwarten, dass transplantierte inaktive C. allata nicht aktiv werden können. Zur Abklärung dieser Frage wurden C. allata aus letzten Nymphenstadien verschiedenen Alters in das Abdomen trächtiger Weibchen implantiert. Im letzten Nymphenstadium sind die C. allata bis etwa zum 8. oder 9. Tag aktiv. In der folgenden Mitoseperiode, die sich bis zum 22. Tag des letzten Stadiums ausdehnen kann, werden die C. allata inaktiv. Nach unterschiedlicher Versuchsdauer zeigte sich nun folgendes Ergebnis (Tabelle 3). Aktive C. allata blieben während der Versuchsdauer aktiv. Infolge der Aktivität der nymphalen C. allata reiften in den Wirtsovarien die Eier. Nymphale C. allata können also im Adultmilieu gonadotropes Hormon produzieren. Wurden C. allata transplantiert, die sich in der Mitoseperiode befanden, so hörten die Mitosen

auf, die Transplantate wurden ein wenig aktiver und infolgedessen reiften Eier in den Ovarien. Wurde hingegen C. allata nach beendeter Mitoseperiode in trächtige Weibchen implantiert, so wurden sie auch nach langer Versuchsdauer nur schwach aktiv. Bei allen Versuchstieren blieben, wie zu erwarten war, die körpereigenen C. allata inaktiv. Diese Versuche zeigen, dass ein inaktives C. allatum

TABELLE 3—IMPLANTATION VON JUVENILEN CORPORA ALLATA IN TRÄCHTIGE WEIBCHEN

Alter der Implantate. Tage letztes Stadium	Anzahl der implantierten C. allata	Fixiert Tage nach Implantation	Zustand der Implantate bei Versuchsende Kernanzahl pro 1 mm³ (Millionen)		Länge der grössten Oocyten im Wirtsovar. (mm)
6	4	12	0,32	0,45	1,87
			0,39	0,32	
15	4	34	0,55	0,62	4,00
			0,58	0,63	
20	2	24	0,71	0,77	0,71
25	6	13	0,77	0,70	0,71
			0,78	0,78	
			0,78	*	
30	2	23	0,71	0,73	0,85

* Ein C. allatum konnte nicht mehr gefunden werden.

ohne Verbindung zum Unterschlundganglion nicht aktiv werden kann. Möglicherweise wird auch ein aktives C. allatum bei langer Versuchsdauer inaktiv, da die stimulierende Wirkung fehlt. Das Versuchsergebnis erklärt vielleicht den Befund, dass nach Implantation jugendlicher C. allata bei *Dixippus* und *Rhodnius* nicht fortlaufend extranymphale Häutungen erfolgen (PFLUGFELDER, 1940; WIGGLESWORTH, 1936), da die C. allata nach einiger Zeit inaktiv werden.

Die C. allata werden also durch zwei Nerven gesteuert: durch hemmende Fasern aus dem Protocerebrum und durch fördernde Fasern aus dem Suboesophagialganglion. Die Eier im Uterus wirken nicht direkt auf die C. allata ein. Unter ihrem Einfluss hemmt ein Zentrum im Gehirn die C. allata-Funktion.

Der Einfluss des Eikokons auf Gehirn und Corpora allata

Nach der Entnahme der Eier aus dem Uterus werden die C. allata rasch aktiv, in den Ovarien reifen die Oocyten (Tabelle 1) und nach durchschnittlich 43 Tagen erfolgt eine Ovulation. Die Eier im Uterus haben also eine hemmende Wirkung auf die C. allata und auf die Eireifung, und diese Wirkung muss über das Gehirn gehen.

Es stellt sich nun die Frage, ob die Eier im Uterus auf nervösem Weg auf das Gehirn einwirken oder ob ihr Einfluss ein humoraler ist (LÜSCHER und ENGELMANN, 1955).

Um die Frage nach der nervösen Beeinflussung des Gehirns abzuklären, wurden die Bauchmarkkonnektive zwischen dem 4. und 5. Abdominalganglion

durchschnitten (Tabelle 4). Es trat eine sofortige Aktivierung der C. allata ein. Infolgedessen begannen in den Ovarien die grössten Oocyten ein wenig zu wachsen. Bei einer längeren Versuchsdauer zeigte sich jedoch, dass die Aktivierung der C. allata nicht weiter ging, sondern stationär blieb.

TABELLE 4—DURCHTRENNEN DES BAUCHMARKS BEI TRÄCHTIGEN WEIBCHEN

	Operation Tage nach Ovulation	Fixierung		C. allatum		Ovar Eilängen (mm)
		Tage nach Operation	Tage nach Ovulation	Volumen (Millionen μ^3)	Kernanzahl pro 1 mm^3 (Millionen)	
a. Eipaket bleibt im Uterus	25	31	6	4,72	0,68	0,64
	19	11	30	4,17	0,77	0,97
	25	16	41	5,08	0,48	0,81
	20	20	40	4,12	0,86	0,93
	30	30	60	5,34*	0,72*	0,90
	12	33	45	3,80	0,72	0,79
Total 6 Tiere	12–30	6–33	30–60	4,54 ± 0,60	0,71 ± 0,13	0,84 ± 0,12
b. Bei Entfernen des Eipaketes nach 5 Tagen	33	14(9)†	42	7,32	0,40	1,74
	37	14(9)†	46	6,95	0,40	1,16
	55	14(9)†	64	8,05	0,26	3,80
	21	18(13)†	34	6,13	0,33	0,98
	38	18(13)†	51	5,32	0,42	0,98
Total 5 Tiere	21–55	14–18	34–64	6,75 ± 1,07	0,36 ± 0,06	1,72 ± 1,20

* Es wurde nur ein Bauchmarkkonnektiv durchtrennt.
† In Klammern Tage nach Entfernen des Eipaketes.

Wenn nun tatsächlich die Anwesenheit von Eiern im Uterus dem Gehirn durch das Bauchmark "mitgeteilt" würde, so dürfte eine nachträgliche Entfernung des Eikokons keinen weiteren Einfluss mehr auf die C. allata haben. Um diese Frage zu prüfen, wurde der Kokon 5 Tage nach einer Bauchmarkdurchtrennung entfernt. Die C. allata zeigten aber nach dieser Operation eine stärkere Aktivität (Tabelle 4). Der Reiz ist also trotz der fehlenden Nervenverbindung dem Gehirn "mitgeteilt" worden. Es ist also anzunehmen, dass durch die Bauchmarkdurchtrennung lediglich infolge unspezifischer Reizung die nervöse Hemmung der C. allata vom Gehirn aus für kurze Zeit gestört oder aufgehoben wird. Die Eier wirken nicht auf nervösem Weg auf das Gehirn ein.

Zur Prüfung der Frage nach einer stofflichen Einwirkung wurden die Eier aus dem Uterus entnommen und sofort in die Körperhöhle des Abdomens implantiert (Tabelle 5a). Zwischen zwei Segmenten wurde die Intersegmentalhaut aufgeschnitten und das Implantat—etwa die Hälfte eines Kokons—in das Abdomen geschoben. Die Wunde wurde mit Paraffin verschlossen. Die Untersuchung der

C. allata nach einer Versuchsdauer von 10 Tagen zeigte eine geringe Plasmazunazme in den Drüsen gegenüber einer normalen Trächtigkeit. Diese Aktivität entsprach aber nicht einer solchen wie sie bei einer Entnahme des Kokons zu beobachten ist (Tabelle 1b). Die Eier üben ihre Wirkung auf das Gehirn auch dann aus, wenn sie sich in der Leibeshöhle befinden und der Uterus leer ist. Vom Eipaket geht also

TABELLE 5—IMPLANTATIONSVERSUCHE NACH ENTFERNEN DES EIPAKETES

Implantat	Operation Tage nach Ovulation	Fixierung		C. allatum		Ovar Eilängen (mm)
		Tage nach Operation	Tage nach Ovulation	Volumen (Millionen μ^3)	Kernanzahl pro 1 mm^3 (Millionen)	
a. Eier	31	10	41	3,75	0,78	0,67
	35	10	45	3,15	0,88	0,67
	33	10	43	2,75	0,78	0,70
	23	14	37	5,23	0,61	0,86
	25	14	39	4,80	0,67	0,70
	25	14	37	4,13	0,78	0,75
Total 6 Tiere	23–35	10–14	37–45	3,96 ± 0,93	0,74 ± 0,10	0,72 ± 0,07
b. Paraffinblöcke	29	10	39	8,10	0,48	0,77
	29	10	39	6,37	0,48	0,79
	31	15	46	9,20	0,34	1,17
	32	15	47	7,35	0,46	0,85
	36	15	51	6,12	0,37	2,00
	35	15	50	5,75	0,41	1,59
Total 6 Tiere	29–36	10–15	39–51	7,15 ± 1,32	0,42 ± 0,06	1,19 ± 0,50
c. Muskulatur	39	12	51	7,50	0,40	0,94
	36	12	48	4,73	0,39	0,86
	34	12	46	5,57	0,49	0,82
	37	12	49	4,48	0,51	0,98
	30	13	43	6,35	0,42	0,99
	30	13	43	4,19	0,51	0,89
Total 6 Tiere	30–39	12–13	43–51	5,45 ± 1,81	0,46 ± 0,05	0,91 ± 0,07
d. Agarblöcke	34	14	48	3,90	0,51	1,02
	32	13	45	2,72	0,72	0,91
	29	13	42	10,65	0,37	0,89
	32	14	46	4,32	0,58	1,00
	33	13	46	5,93	0,49	1,32
Total 5 Tiere	29–34	13–14	42–48	5,51 ± 3,12	0,52 ± 0,13	1,03 ± 0,17
e. Ovulationskokon getrocknet bei 120°C	28	13	41	2,61	0,88	0,81
	31	13	44	13,10	0,33	1,32
	30	13	43	6,70	0,37	1,05
Total 3 Tiere	28–31	13	41–44	7,47 ± 5,25	0,52 ± 0,31	1,06 ± 0,25

eine stoffliche Wirkung aus, welche das Gehirn zur nervösen Hemmung der C. allata veranlasst. Vierzehn Tage nach der Operation liess die Hemmwirkung nach. Zu dieser Zeit waren die Eier nicht mehr am Leben. Möglicherweise geben nur lebende Eier den Wirkstoff in genügender Menge ab.

Um die Möglichkeiten einer mechanischen Druckwirkung auf das Abdomen, oder die Wirkung unspezifischer Stoffe, die beim Abbau von Geweben in der Leibeshöhle entstehen, auszuschliessen, wurden nach Entfernen des Eikokons Paraffinblöcke, Muskeln oder Agarblöcke in der Grösse eines halben Eipaketes implantiert (Tabelle 5b–d). In allen diesen Versuchen wurden die C. allata aktiv. Am besten zeigte sich bei der Implantierung von Paraffinblöcken (Tabelle 5b), dass eine mechanische Druckwirkung die Hemmung der C. allata nicht verursachen kann. Nach Implantation von Agarblöcken und Muskeln (aus den Hüften männlicher Schaben) wurden ganz unterschiedliche Ergebnisse erzielt (Tabelle 5c, d). In einigen Fällen ergab sich eine beinahe maximale Aktivität. Hier war sicher keine Hemmwirkung vorhanden. Gehemmte C. allata lassen nicht unbedingt auf Vorhandensein von Hemmstoffen schliessen, da für die Hemmung noch andere Faktoren eine Rolle spielen können. Es ist zum Beispiel daran zu denken, dass infolge der Operation die Tiere manchmal keine Nahrung zu sich nehmen (Tabelle 1c). Ganz allgemein gilt für diese Versuche, dass für den Beweis einer Inaktivität der C. allata mehr Versuchstiere benötigt werden, als für den Beweis der Aktivität. In einer Versuchsserie weisen schon 1–2 Tiere mit aktiven Drüsen auf ein Versagen des Hemmechanismus hin.

Man kann sich nun weiter fragen, welcher Art dieser Hemmstoff sei. Die Hemmung der C. allata durch das Sekret einer embryonalen Drüse ist nicht anzunehmen, da offenbar das ungefurchte Ei gegen Ende der Eireifung schon eine schwache Wirkung auf die C. allata ausübt (Abb. 3). Der Umstand, dass gegen Ende der Trächtigkeit eine geringe Aktivierung der C. allata beobachtbar ist, weist darauf hin, dass die Hemmwirkung vom Dottermaterial, das im Laufe der Embryonalentwicklung mengenmässig abnimmt, ausgehen könnte. Hierfür spricht auch folgende Beobachtung. Bei einigen Weibchen, bei denen der Eikokon entfernt wurde, reiften in den Ovarien keine neuen Eier, da die C. allata inaktiv blieben (Tabelle 6). Die Sektion ergab, dass bei der vorausgegangenen Ovulation einige Eier in den Ovarien zurückgeblieben waren und dort resorbiert wurden. Bei der Resorption des Dotters wurde vermutlich ein Stoff frei, der die Allatazentren im Gehirn veranlasste, die C. allata in ihrer gonadotropen Funktion zu hemmen. Die Beobachtung, dass nicht alle Eier aus den Ovarien bei der Bildung des Kokons abgehen, wird besonders häufig in den Fällen gemacht, in denen kein richtiger Kokon geformt wird. Dies kommt vor allem bei Jungfräulichkeit vor (ENGELMANN, 1957). Einen eingehende Untersuchung von Tieren, deren Ovarien Eier enthielten, die sich in Resorption befanden (Tabelle 6), zeigte eine deutliche Hemmung der C. allata-Funktion. Erfolgte in den Ovarien gegen Ende der Resorption bereits wieder eine erneute Reifung der nächsten Eier, so reiften in Ovariolen, die Resorptionseier enthielten, keine Oocyten. Hier scheint auch eine direkte Hemmung der Eireifung in den Ovariolen zu bestehen, solange sich Reste

eines alten Eies darin befinden. Die Resorption eines zurückgeblieben Eies ist nach etwa 70–90 Tagen beendet, und es kann sodann eine neue Eireifung beginnen.

TABELLE 6—CORPORA ALLATA BEI RESORPTION VON EIERN IN DEN OVARIEN

Alter der Resorptionseier in Tagen	Anzahl der Eier in Resorption	C. allatum		Ovar Eilängen (mm)
		Volumen (Millionen μ^3)	Kernanzahl pro 1 mm^3 (Millionen)	
25	1	3,50	0,62	0,73*
59	4	3,18	0,78	0,73†
50	7	1,54	0,99	0,81‡
26	4	3,11	0,84	0,85
35	40	6,25	0,65	0,66
35	40	2,85	0,71	0,68
40	40	4,39	0,73	0,66
65	40	3,74	0,89	0,56
55	5	5,65	0,67	0,75
55	4	6,14	0,51	0,88
71	4	3,77	0,48	1,35§
84	5	10,60	0,36	2,20§
127	4	5,95	0,37	1,00§
83	5	4,54	0,87	0,67
90	9	4,94	0,56	0,70

* 9 Tage nach Kokonentfernen.
† 14 Tage nach Kokonentfernen.
‡ 12 Tage nach Kokonentfernen.
§ Ein Wachstum der Eier in Ovariolen, die Resorptionseier enthalten, erfolgt nicht.

Der von den Eiern produzierte Hemmstoff scheint nicht hitzebeständig zu sein. Wird dem Weibchen nach Entnahme des Eipakets ein bei 120°C getrocknetes Eipaket implantiert, so werden die C. allata nicht gehemmt (Tabelle 5e).

Die Implantations—und Explantationsversuche zeigen, dass die Wirkung des Eipaketes im Uterus auf das Gehirn und die C. allata stofflicher Natur sein muss.

DISKUSSION

Die experimentelle Analyse der Abhängigkeit der Ovarfunktion vom Trächtigkeitszyklus bei *Leucophaea* hat folgendes Funktionsschema ergeben (Abb. 7).

Die Eier im Uterus geben einen Stoff ab, der direkt oder indirekt auf das Gehirn einwirkt. Dadurch werden die Allatum-Zentren im Protocerebrum veranlasst, auf nervösem Wege die C. allata zu hemmen. Diese geben kein oder nur in ungenügenden Mengen gonadotropes Hormon ab. Infolgedessen unterbleibt das mit der Einlagerung von Dotter verbundene Wachstum der Eier in den Ovarien und die Anhangsdrüsen bleiben inaktiv. Nach einer Geburt oder nach experimenteller Entfernung des Eipaketes fällt der stoffliche Einfluss der Eier weg, die Allatum-Zentren im Protocerebrum sind nicht mehr beeinflusst und hemmen die

C. allata-Tätigkeit nicht mehr. Die C. allata werden infolge eines stimulierenden Nerveneinflusses vom Suboesophagialganglion aktiviert und produzieren gonadotropes Hormon (Eireifungshormon), das auf die Ovarien und die Anhangsdrüsen einwirkt. In den Ovarien wachsen unter Einlagerung von Dotter Eier heran und die Anhangsdrüsen produzieren Sekret, das später zur Bildung des Eikokons dient.

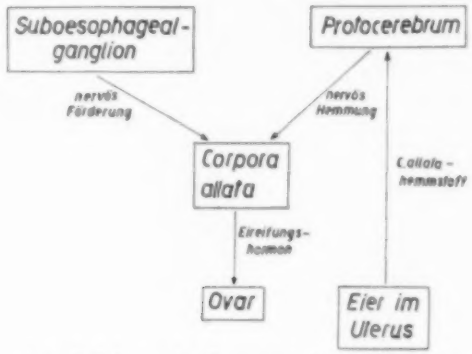

ABB. 7. Schema der Steuerung der Ovarfunktion.

Es soll nun im Folgenden geprüft werden, ob sich aus dieser Untersuchung neue Befunde von allgemeiner Bedeutung ergeben.

Die Wirkung der Eier auf die Corpora allata

Bei verschiedenen Insekten konnte eine Wirkung des Ovars auf die C. allata nachgewiesen werden. Im allgemeinen werden nach Kastration die C. allata vergrössert, sodass oft von einer Hypertrophie gesprochen wird (BODENSTEIN, 1947; DAY, 1943; PFEIFFER, 1945; THOMSEN, 1940, 1955; VOGT, 1942; WIGGLESWORTH, 1948). Die C. allata werden also von den Ovarien gehemmt. Im Falle von *Leucophaea* wirken hauptsächlich die Eier im Uterus hemmend auf die C. allata.*

Uber die Natur des Wirkstoffs, der von dotterhaltigen Eiern ausgeht, lässt sich vorläufig nur sagen, dass er hitzelabil ist. Möglicherweise handelt es sich um ein Abbauprodukt, das bei der Dotterresorption entsteht.

Der Hemmstoff der Eier wirkt sicher nicht direkt auf die C. allata ein. Schon die Nervdurchtrennungsversuche von SCHARRER (1952a) sprechen für eine Beeinflussung der C. allata durch das Gehirn. Auch bei *Sarcophaga* führte ein Durchtrennen der Ringdrüseninnervierung zu einer Vergrösserung der C. allata (DAY, 1943). Die eigenen Durchtrennungs- und Ausschaltversuche haben gezeigt, dass im Protocerebrum gelegene Allatum-Zentren unter dem Einfluss des Wirkstoffs stehen müssen. Ob allerdings der Wirkstoff direkt auf diese Zentren bzw. auf das Gehirn einwirkt, ist sehr fraglich. Im Falle der Auslösung der Metamorphose konnte gezeigt werden, dass die Pericardialzellen durch einen stofflichen Faktor das entsprechende Gehirnzentrum zur Hemmung der C. allata

* Ob auch die reifen Eier im Ovar hemmend wirken, wird noch zu prüfen sein. Untersuchungen darüber werden gegenwärtig durchgeführt.

veranlassen können (ENGELMANN und LÜSCHER, 1956). Möglicherweise produzieren die Pericardialzellen zur Zeit der Metamorphose einen dem Wirkstoff der Eier vergleichbaren Stoff. Es ist aber nicht ausgeschlossen, dass die Wirkung der Eier im Uterus über die Pericardialzellen geht. Ferner besteht die Möglichkeit, dass noch weitere Systeme dazwischengeschaltet sind.

Die Wirkung des Gehirns auf die Corpora allata

Es ist für verschiedene Insekten nachgewiesen worden, dass nach Durchtrennung der Nervenverbindung zwischen Gehirn und C. allata nicht nur eine Vergrösserung der letzteren erfolgt (für *Leucophaea* durch SCHARRER (1952), ENGELMANN und LÜSCHER (1957) und für *Sarcophaga* durch DAY (1943)), sondern dass die C. allata dadurch aktiviert werden (überzählige Larvenhäutungen bei *Dixippus* (PFLUGFELDER, briefliche Mitteilung (1956)) und bei *Leucophaea* (SCHARRER, 1952; ENGELMANN und LÜSCHER, 1957), oder Verhinderung der Verpuppung bei Dipteren (BURTT, 1938)). Es hat sich jetzt gezeigt, dass die C. allata nicht nur in Bezug auf die Juvenilhormonproduktion, sondern auch in Bezug auf die Produktion gonadotropen Hormons nach dieser Operation aktiv werden. SCHARRER lässt die Frage offen, ob der Einfluss vom Gehirn auf die C. allata durch das Neurosekret oder auf nervösem Wege ausgeübt wird. Würde man annehmen, dass das Neurosekret für die Steuerung der C. allata verantwortlich sei, so müsste dieses die C. allata hemmen, da die Hemmung nach Nervdurchtrennung aufgehoben wird. Wir haben aber festgestellt, dass bei Fehlen von Neurosekret die C. allata gar nicht aktiv werden können. Wir hätten dann die paradoxe Situation, dass der gleiche Wirkstoff, der für eine Aktivierung unbedingt erforderlich ist, diese Aktivierung auch verhindern kann. Dies ist sehr unwahrscheinlich. Zudem haben die eigenen Versuche gezeigt, dass durch einseitige Ausschaltung der neurosekretorischen Zellen die C. allata unbeeinflusst bleiben, dass aber durch einseitige Ausschaltung eines nervösen Zentrums im Protocerebrum das C. allatum der gleichen Seite enthemmt wird.

Nach Durchtrennen des medianen Cardiacum-Nerven wird die C. allata-Hemmung während der Trächtigkeit nur dann aufgehoben, wenn die Nervenverbindungen zwischen C. allata und Suboesophagialganglion intakt bleiben. Bei *Dytiscus* (JOLY, 1945) atrophieren nach einer Durchtrennung der C. cardiaca die C. allata. Möglicherweise wurden hier bei der Operation die Nerven zum Unterschlundganglion mitdurchtrennt. Transplantierte C. allata bleiben etwa in dem Zustand in dem sie bei der Operation waren; inaktive Drüsen werden nur sehr schwach aktiv und aktive Drüsen bleiben einige Zeit aktiv (Tabelle 3). Bei *Dixippus* degenerieren transplantierte C. allata längere Zeit nach der Operation (PFLUGFELDER, 1939, 1940); bei Bienen geht von implantierten Königinnen-C. allata keine gonadotrope Wirkung aus (MÜSSBICHLER, 1952) und bei *Rhodnius* werden durch transplantierte C. allata nur 1–2 Extrahäutungen erzielt (WIGGLESWORTH, 1948). Es scheint dass die C. allata nicht dauernd funktionell bleiben, wenn sie von allen Nervenverbindungen gelöst sind. Wahrscheinlich werden vom Unterschlundganglion aus die C. allata stimuliert.

Die Funktion der C. allata scheint also ganz allgemein durch zwei Nerven reguliert zu werden, die antagonistisch wirken. Vom Unterschlundganglion wird die Funktion stimuliert. Hier liegt vielleicht eine Dauerwirkung vor, wobei nicht sichergestellt ist, ob es sich um eine nervöse Stimulierung oder um eine tropische Wirkung handelt. Vom Gehirn aus wird die C. allata-Funktion gehemmt, vermutlich durch Nervenimpulse; sicher nicht direkt durch das in den Nerven fliessende Neurosekret. Die Hemmung kann zeitweise durch den Ausfall stofflicher Einflüsse aufgehoben werden.

ZUSAMMENFASSUNG

(1) Nach der Metamorphose reifen die Eier in den Ovarien in durchschnittlich 33 Tagen zur Ovulationsgrösse heran. Während der Trächtigkeit wird kein Dotter in die Oocyten eingelagert. Sofort nach der Geburt der Jungen werden die Ovarien wieder aktiv und die Oocyten reifen unter Dottereinlagerung in etwa 22 Tagen. Während der Eireifungsperiode sind die Anhangsdrüsen des Geschlechtsapparates aktiv und produzieren grosse Mengen Sekret, das zur Kokonbildung dient.

(2) Während jeder Eireifungsperiode sind die C. allata aktiv; in der Trächtigkeitsperiode jedoch inaktiv. Sie haben also parallel mit den Ovarien einen Aktivitätszyklus.

(3) Die Aktivierung der Ovarien und Anhangsdrüsen erfolgt unabhängig von einander durch die C. allata.

(4) Die C. allata werden während der Trächtigkeit vom Protocerebrum aus gehemmt. Die Hemmung erfolgt nervös durch den medianen Cardiacum-Nerv.

(5) Das Hemmzentrum für die C. allata liegt wahrscheinlich im Protocerebrum in der Nähe der Protocerebralbrücke oder des Zentralkörpers.

(6) Eine Nervenverbindung vom Unterschlundganglion zum C. allatum muss intakt bleiben, damit eine Aktivierung der C. allata möglich ist. Es ist zu vermuten, dass dieser Nerv stimulierend wirkt.

(7) Transplantierte C. allata verbleiben etwa in dem Aktivitätszustand, in dem sie sich bei der Explantation befinden.

(8) Wird ein Eipaket aus dem Uterus entnommen, so werden die, C. allata rasch aktiviert und infolge dieser Aktivierung reifen in den Ovarien die nächsten Eier. Hungern die Weibchen jedoch, so bleiben die C. allata inaktiv oder werden nur sehr schwach aktiv.

(9) In die Leibeshöhle implantierte Eier hemmen die C. allata fast so stark, wie wenn sie sich im Uterus befänden. Die Eier im Uterus geben also einen Stoff ab, der die C. allata durch die Beeinflussung spezieller Zentren im Gehirn hemmt.

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PROTEOLYTIC ENZYMES IN VARIOUS EMBRYONIC STAGES OF THE EGGS OF *LOCUSTA MIGRATORIA MIGRATORIOIDES* (R. AND F.)

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Abstract—The action of homogenates of eggs of *Locusta migratoria migratorioides* (R. and F.) in various stages of development on casein and on leucylglycylglycine at various pH values was investigated. The pH values of the homogenates of eggs in different stages were found to vary from 6·0 to 6·6. In no case was hydrolysis of casein or leucylglycylglycine obtained with freshly laid eggs. At pH 5·6 a distinct cleavage of casein was obtained with batches of eggs varying from stage VIII to stage XII (i.e. during the polypod stage and the beginning of the oligopod stage). This action increased towards the end of development. At pH 7·8 a distinct action on casein appeared occasionally with batches of eggs varying from stage X to stage XX (i.e. from the advanced polypod stage to the stage when the head of the embryo reaches the anterior end of the egg after blastokinesis). This action was always found in the batches with eggs from stages XVIII to XXIII (i.e. immediately after the end of blastokinesis to shortly before hatching) and markedly increased towards hatching. At pH 6·5 a slight action on casein appeared with the batches containing eggs of stages VIII–XII. The cleavage became distinct with stages X–XX, and increased further towards the end of development. A distinct action on leucylglycylglycine, measured mainly at pH 7·8, occurred with stages III–XI and a greater one with eggs of all higher stages.

It is concluded from the results that the homogenates of the eggs contain at least two kinds of endopeptidases.

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IT HAS been shown that a glycerine extract of the eggs of the Desert Locust, *Schistocerca gregaria* (Forskål), during the first few days of development attacks casein at pH 8 only to a very slight degree, if at all, while towards the end of the development, and especially shortly before hatching, hydrolysis is clearly pronounced (KUK-MEIRI, SHULOV and LICHTENSTEIN, 1954).

It therefore seemed of interest to study the correlation between the different stages of development of locust eggs and the proteolytic activity.

For this study, *Locusta migratoria migratorioides* (R. and F.) was chosen, and the action of homogenates of these eggs, at different embryonic stages (SHULOV and PENER, 1957), on casein and leucylglycylglycine was investigated at various pH values.

MATERIAL AND METHODS

The continuous breeding of *L. m. migratorioides* was started with eggs which were kindly supplied in 1953 by the Anti-Locust Research Centre, London. The locusts were kept under crowded conditions and were of distinct *gregaria* phase.

The experiments described below were carried out with eggs laid between April 1955 and February 1956.

The locusts laid their egg pods in tubes filled with moist sand (HUNTER-JONES, 1956). The tubes were checked and the egg pods removed daily. The eggs were considered to be 0 days old at the time of removal.* At fixed intervals, 1–10 of the egg pods were opened. One out of each six eggs in each pod was killed in a fixing fluid (Bouin), and its embryonic stage was determined. The other five were used for enzymatic experiments. The number of eggs used in one series of experiments varied from 14 to 240.

The eggs taken for the experiments were weighed, washed, and ground in a mortar with water to a fine suspension which was subsequently diluted in order to make its final volume (in ml) numerically equal to 10 times the weight of the eggs. An equal volume of 0·1 phosphate buffer at the appropriate pH was added.

A casein solution (1 per cent) was prepared by dissolving the protein in excess of 0·1 in NaOH and by adding HCl drop by drop to the desired pH. The volume was made up by adding the appropriate phosphate buffer.

DL-leucylglycylglycine was used in 2 per cent aqueous solution.

For testing the proteinase activity by Anson's method (NORTHROP, KUNITZ and HERRIOTT, 1948) 1 ml of each enzyme and of the casein solution were mixed. Controls run simultaneously did not contain substrate or enzyme. The reaction mixture was kept at 36°C for about 24 hr under toluene, 3 ml of 5 per cent trichloracetic acid was added, and the mixture was kept overnight in the cold (+ 5°C) and filtered. To 1 ml of the filtrate were added 8 ml of 0·5 N NaOH and 3 ml of phenol reagent of Folin and Ciocalteou. The colour produced was measured in the Klett-Sommerson photoelectric colorimeter using filter 69. The colour values obtained with corresponding mixtures at the beginning of the experiments as well as the controls were subtracted. In some of the experiments a part of the filtrate obtained on addition of trichloracetic acid was also tested, after appropriate dilution with water, in the Beckman UV Spectrophotometer at 273 mμ (HOLIDAY, 1936).

The action of leucylglycylglycine was measured as follows: to 5 ml of leucylglycylglycine solution were added 5 ml of 0·1 M phosphate buffer and 2·5 ml of enzyme solution. Samples of 5 ml were tested by Sørensen formol titration method at the beginning, and again after standing for 24 hr under toluene at 36°C. Controls were run with enzyme or substrate omitted.

The pH values were measured with the aid of a Beckman glass-electrode pH meter.

RESULTS

In Table 1 are presented the results of experiments on the action of the egg homogenates at different stages of embryonic development on casein and on leucylglycylglycine at various pH values. The table shows that the morphological changes of a developing embryo are followed by qualitative as well as quantitative variations in the proteolytic activities.

* The pods were kept in moist sand at 27 ± 1°C.

Action on casein at pH 7·8. No significant action could be detected in the batches of eggs in stages 0–XIV (i.e. from the beginning of the development up to the last morphological stage before katatrepsis). However, in the batches of eggs ranging from stage X to stage XX (i.e. when the thoracic appendages of the embryo in a polypod stage begin turning medially until the head of the late oligopod embryo reaches the anterior end of the egg) proteolytic action was occasionally found. The batches of eggs of stages XVIII–XXIII (i.e. from the stage when half of the egg is occupied by the embryo after the end of blastokinesis until hatching) always showed proteolytic action. This action markedly increased towards the end of the development and at hatching.

Action on casein at pH 5·6. No action was found at the beginning of development (stage 0), but a distinct action could be detected at stages VIII–XII (during the polypod and beginning of the late oligopod stages). This action increased in the subsequent stages, the rate of increase being higher towards the end of development and at hatching.

Action on casein at pH 6·5. No action was found at stage 0 and only a very slight one, if any, at stages VIII–XII. This action increased and became distinct in the batches containing stages X–XX, increasing again towards the end of development and at hatching.

Action on leucylglycylglycine at pH 7·8. No action was found at stages 0–I (i.e. up to 2 days). A distinct one, however, was found with stages III–XI and there was an increase from XI until XIX, followed by a slight decrease towards the end of the development.

As may be seen from Table 1, no endopeptidase activity (cleavage of casein) could be detected at the beginning of the development, under the conditions of these experiments. At stages VIII–XII an action is present at pH 5·6 but no action was found at pH 7·8. During stages X–XX there is evidence of action at pH 5·6 and occasionally at pH 7·8. From XVIII to XXIII action was found at both pH values, the cleavage at the lower value being predominant. Shortly before hatching and at hatching the cleavage increased at both pH values; in this case, however, the hydrolysis was not more pronounced in the acid medium.

Several measurements carried out with the aid of the Beckman Spectrophotometer (wavelength 273 mμ) gave results of the same order of magnitude as those obtained by the colorimetric method.

Pre-incubation of the egg homogenates for 2 and 24 hr resulted in only a slight decrease, if any, in the cleavage of casein. These experiments were carried out at pH 7·8 with eggs of stages XX–XXIII.

Cleavage of casein was also measured after an incubation of 72 hr at pH 7·8, with the eggs of stages XI–XVIII, XVII–XX, and XXI–XXIII. In all these cases the cleavage was 2 to 3 times stronger than that obtained after 24 hr.

The pH values of the egg homogenates in water varied from 6 to 6·6 (Table 2). They were measured in 7 batches of eggs, each taken from several pods.

DISCUSSION

The results presented above suggest that in the developing egg there are at least two kinds of endopeptidases. One measured at pH 7·8 is probably trypsin-like, whereas the other, which is responsible for the cleavage of the casein at pH 5·6,

TABLE 1—ACTION OF HOMOGENATES OF *Locusta migratoria migratorioides* EGGS AT DIFFERENT STAGES (The figures for the action on casein represent the increase in Klett-Sommerson colorimeter reading and

Serial number of experiment	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Time of experiment	May 1955	Jan. 1956	Feb. 1956	Aug. 1955	Sept. 1955	July 1955	Feb. 1956	Feb. 1956	July 1955	July 1955	Aug. 1955	Jan. 1956	Jan. 1956	July 1955	Sept. 1955
Number of eggs tested	106	103	95	39	225	69	80	92	25	75	39	92	80	90	236
Average weight of one egg (mg)	7·8	7·6	6·8	9·4	9·4	8·6	9·05	9·7	16·1	10·5	15·9	13·2	15·2	17·2	13·5
Age of the eggs in days	0	0	0	2	4, 5	5	5	5	6	7	8	7	7	7	7, 8
Embryonic stage (SHULOV and PENER, 1957)	0	0	0	0–I	III–XI	VI–IX	VIII–IX	X–XII	XI–XIII	XII–XIV	XII–XIII	XI–XVIII	XI–XVIII	X–XVIII	X–XIX
Action on casein															
pH 7·8 ± 0·1	−2	+7	0	—	−6	+17	+10	+7	+2	−12	−1	+67	+60	+10	+7
pH 6·5 ± 0·1	—	+2	—	—	+4	—	+28	+23	—	—	—	+64	+58	—	+55
pH 5·6 ± 0·1	—	−9	+9	—	—	—	+81	+83	—	—	—	+133	+120	—	—
Action on leucylglycylglycine															
pH 7·8 ± 0·1	0·15	—	—	0·0	0·8	1·2	—	—	1·4	1·9	1·25	—	—	1·8	1·5
pH 6·5 ± 0·1	—	—	—	—	0·25	—	—	—	—	—	—	—	—	—	0·2

probably belongs to the cathepsine-like type of enzymes. The cleavage of the casein found at pH 6·5 may be caused by the weak action of one of the enzymes or by both enzymes reacting together.

As pointed out in our previous paper (KUK-MEIRI, SHULOV and LICHTENSTEIN, 1954), there is a possibility of the existence of a correlation in time between the

TABLE 2—pH VALUES OF HOMOGENATES OF EGGS OF *Locusta migratoria migratorioides* AT VARIOUS EMBRYONIC STAGES

Serial number of experiments in Table 1	Age of eggs (days)	Embryonic stages	pH values (± 0·1)
6	4 and 5	III–XI	6·0
14	7	XI–XVIII	6·2
16	7 and 8	X–XIX	6·3
17	7	XI–XX	6·6
18	8	XVII–XVIII	6·4
26	13	XX–XXII	6·5
28	15	XXI–XXIII	6·5

appearance of proteolytic enzymes and certain stages of the development of the eggs of *Schistocerca gregaria*. The present paper shows that there is a correlation in time between the appearance of proteases and the different stages of egg development of *Locusta migratoria migratorioides*.

OF EMBRYONIC DEVELOPMENT ON CASEIN AND LEUCYLGLYCYLGLYCINE AT VARIOUS pH VALUES
those for the action on leucylglycylglycine represent the increase in titration value in ml of 0·05 N NaOH.)

17	18	19	20	21	22	23	25	26	27	28	29	30	31	32	33
Sept. 1955	Jan. 1956	Feb. 1956	Jan. 1956	July 1955	Jan. 1956	Dec. 1955	July 1955	Jan. 1956	Jan. 1956	Sept. 1955	Feb. 1956	Aug. 1955	Dec. 1955	Feb. 1956	July 1955
200	113	78	77	38	110	85	47	61	170	?	50	25	28	62	14
13·6	15·6	17·9	16·1	19·4	17·8	16·1	18·9	17·05	17·05	?	16·6	15·6	15·7	16·2	18·9
7	8	9	10	11	11	11, 12	13	13	14	15	15	15, 16	16, 17	16	17
XI–XX	XVII–XVIII	XVII–XX	XVIII–XX	XX	XVIII–XXIII	XXI–XXIII	XXI–XXII	XX–XXII	XXI–XXIII	XXI–XXIII	XXIII	XXIII	XXIII-hatching	XXIII-hatching	hatching
+4 +40 —	+39 +32 +114	+70 +53 +153	+68 +55 +130	+74 — —	+45 +53 +142	+102 — —	+44 — —	+124 +79 +155	+138 +94 +149	+41 +48 —	+252 +215 +219	— — —	+170 — —	+183 +134 +218	+300 — —
1·0 0·1	— —	— —	— —	1·1 —	— —	— —	1·1 —	— —	— —	1·05 0·3	— —	1·0 —	— —	— —	— —

The presence of the cathepsin-like endopeptidase in the eggs of *Musca domestica* was recently described by GREENBERG and PARETSKY (1955). Our experiments have shown that the proteolytic activity at pH 5·6 did not appear at the start of the development, but did so at quite early stages (VIII–XII). As found by FRUTON and his collaborators (FRUTON *et al.*, 1951 ; JONES *et al.*, 1952), intracellular animal and plant proteinases exhibit hydrolysing actions at a pH near 5, whereas at higher pH values transpeptidation reactions also occur. The catalysis of transpeptidation reactions is possibly a major function of the intracellular proteinases at physiological pH values (FRUTON and SIMMONDS, 1953). It is possible that the cathepsin-like enzyme found by us is instrumental in a similar way during the development of the locust egg. This suggestion should be considered on the basis of the fact that the pH value of the egg homogenates fluctuates between 6 and 6·6 at various stages of the development (Table 2). Further proof of the suggestion that this enzyme has cathepsin-like properties is furnished by the findings of NORMAN (1954). In his paper on the eggs of *Melanoplus differentialis* he states that the quantity of non-proteinic SH (probably glutathion) increases in the embryo during the development of the eggs. It is well known that glutathion is a natural activator of the cathepsins.

The enzyme responsible for the cleavage of the casein, which has been found at pH 7·8, seems to belong to the trypsin group, well known from the digestive system of insects (DAY and WATERHOUSE, 1953).

ROONWAL (1937), describing the development of the midgut of *Locusta migratoria migratorioides*, pointed out that shortly after the blastokinesis definite groups of cells appear on the ends of both the proctodeal and the stomodeal invaginations which take part in the organization of the midgut. According to his observation (at 33°C), the blastokinesis occurs on the sixth day of the development and the provisional dorsal closure one day later. The definite midgut arises 5 days after the blastokinesis, i.e. 2 days before hatching, the whole development of the egg thus taking 13 days. Our experiments have been carried out at 27°C and the whole development takes 16–17 days (SHULOV and PENER, 1957). There is a slight difference as compared with the findings of HAMILTON (1950), who with the same insect at 26·7°C observed hatching after 15 days. The blastokinesis occurred in our experiments on the seventh–eighth day (at 27°C). The beginning of the activity of the trypsin-like enzyme thus coincides with the beginning of the organization of the midgut after blastokinesis (stage XVIII) in our experiments. The period of the prominent activity of this enzyme takes place simultaneously with the advancing organization of the midgut and reaches the maximum just before hatching and at hatching.

Only a few papers deal with the occurrence of proteolytic enzymes in the eggs of insects. LICHTENSTEIN (1947) obtained cleavage of casein at pH values near 8 with glycerol extracts of the eggs of *Bombyx mori* only a few days before hatching. LICHTENSTEIN, BODENHEIMER and SHULOV (1949) described the action of glycerol extracts of developing eggs of *Dociostaurus maroccanus* kept in moist sand at 27°C. If one compares the results obtained by these authors with the study of BODENHEIMER and SHULOV (1951) on the ecological embryology of this species, it will be seen that a distinct cleavage of casein at pH 8 failed to appear in extracts of eggs prepared before the end of blastokinesis. Here, too, the action on casein markedly increased in extracts made shortly before hatching. A considerable proteolytic action on casein, at pH 8, of the glycerol extracts of the eggs shortly before hatching was also found with *Schistocerca gregaria* (KUK-MEIRI, SHULOV and LICHTENSTEIN, 1954).

It appears from our results and from the preceding investigations that the appearance of a proteinase activity at alkaline pH values is connected with the development of the digestive system.

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ALKALINE PHOSPHATASE IN THE GERM-CELL DETERMINANT OF THE EGG OF *APANTELES*

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Abstract—The germ-cell determinant in the unfertilized egg of *Apanteles glomeratus* L. is a site of alkaline phosphatase activity. In the newly laid egg this enzyme plays an important part in the histolysis of this determinant, resulting in histodifferentiation of that part of the egg where an early segregation of germ cells usually happens. There is an unknown relationship between the products of the histolysis of the germ-cell determinant and those cleavage nuclei which have entered its area, and which become differentiated into the nuclei of the primordial germ cells.

INTRODUCTION

THE germ-cell determinant has been demonstrated in the eggs of many insects, and its presence is always associated with the primordial germ cells. The localization of this inclusion at the posterior pole of the egg of certain parasitic Hymenoptera is clearly observable. Where an early segregation of germ cells has been noticed, this body appears in the cytoplasm of the young oöcyte. After the egg is laid, this inclusion breaks up into granules at an early embryological stage and becomes distributed among the germ cells.

METHOD AND TECHNIQUE

For the demonstration of alkaline phosphatase the calcium-cobalt technique of GOMORI (1946) was used. Neutral-formol smears of the ovarian eggs of *Apanteles glomeratus* L. were treated as follows:

After washing with distilled water, the slides were incubated from 30 min to 6 hr at 37°C in the following medium:

2 per cent Na B glycerophosphate	10 cm³
2 per cent Na diethyl barbiturate	10 cm³
Distilled water	20 cm³
2 per cent calcium chloride	2 cm³
2 per cent magnesium sulphite	1 cm³

After rinsing the slides with running water they were treated with 2 per cent cobalt nitrate for 5 min. After washing carefully in distilled water they were passed to a dilute solution of yellow ammonium sulphide. The slides were washed again in water, dehydrated, cleared in xylol, and mounted.

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For acid phosphatase GOMORI's method was also followed, using sodium B glycerophosphate in the incubating substrate prepared by Professor W. J. E. JESSOP as follows:

Distilled water	6 vol.
Molar acetic acid	1 vol.
Molar acetate	2 vol.
5 per cent lead nitrate	1 vol.
2 per cent Na B glycerophosphate	3 vol.

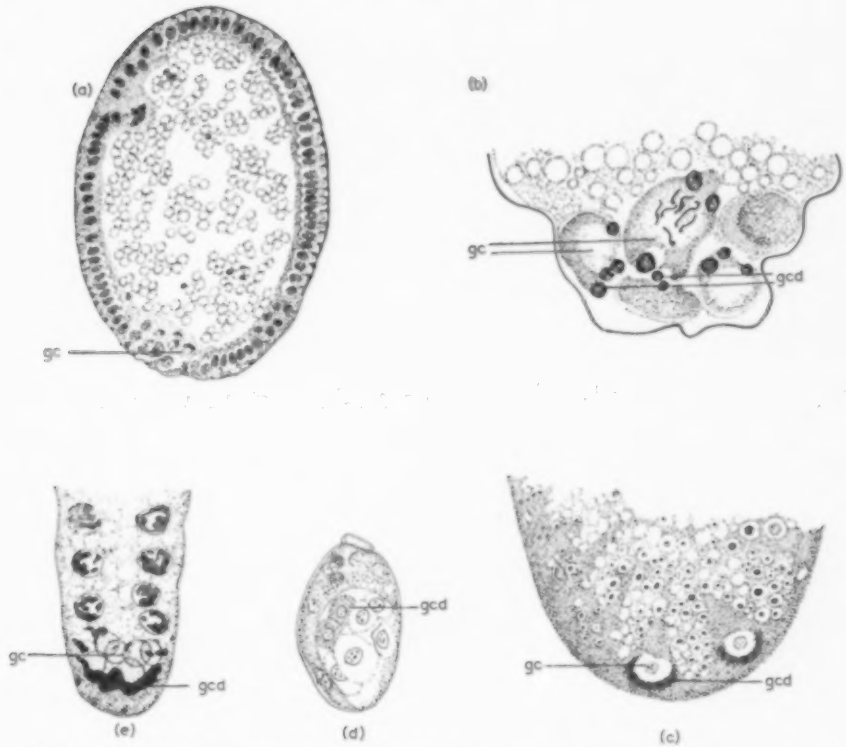

FIG. 1. Section of eggs of *Brachyrhinus* (a), *Sciara* (b), *Calliphora* (c), *Litomastix* (d) (after JOHANNSEN and BUTT), and (e) *Trichogramma* (after GATENBY), showing the dividing germ-cell determinant spread around the germ cells; gc, germ cell; gcd, germ-cell determinant.

After rinsing the slides with water they were placed in the mixture at 37°C. The duration of the incubation period was 2 to 24 hr. The slides were washed and then put in 1 per cent acetic acid. After rinsing in water they were treated with a dilute solution of ammonium hydrosulphide. The slides were then washed in water, dehydrated, cleared in xylol, and mounted.

OBSERVATIONS

The germ-cell determinant in *Apanteles glomeratus* L. arises as a cloud of granules (probably formed of albuminous material) which appear spontaneously in the cytoplasm at one end of the oöcyte (GATENBY, 1920). In the egg, just before oviposition, this inclusion appears in longitudinal section at the posterior pole as a triangular area of granules darkly stained with the iron-haematoxylin (Figs. 2 and 3).

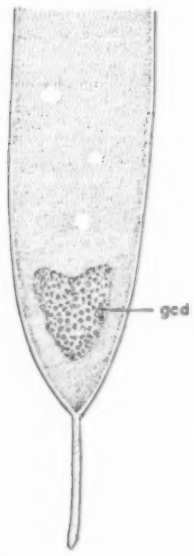

FIG. 3. Longitudinal section through the posterior pole of the mature ovarian egg of *Apanteles* showing the germ-cell determinant.

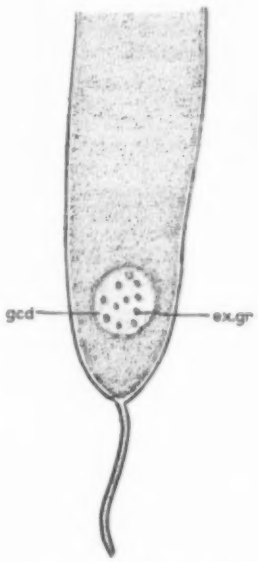

FIG. 4. Posterior pole of *Apanteles* ovarian egg, drawn from a smear, showing the enzyme granules of alkaline phosphatase in the germ-cell determinant.

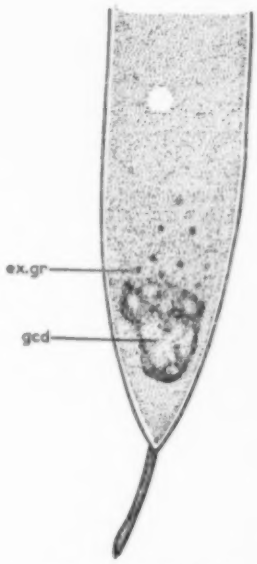

FIG. 5. Longitudinal section through the posterior pole of a newly laid egg showing the germ-cell determinant undergoing histolysis.

ex. gr, extruded granules; ez. gr, enzyme granules; gc, germ-cell; gcd, germ-cell determinant.

The germ-cell determinant in the egg of *Apanteles* shows a positive reaction to the alkaline phosphatase method. The site of the enzyme is clearly seen after 75 min incubation in all the preparations. The general appearance of this inclusion is brownish, but at the site of the enzyme black granules are present (Fig. 4). Comparing the number of the enzyme granules, which are about eleven, with the numerous iron-haematoxylin stained granules of the germ-cell determinant, it seems that the latter are not the site of the enzyme.

Acid phosphatase is not demonstrable in this cytoplasmic inclusion. Even when the incubation period was extended to 36 hr, the germ-cell determinant showed no histochemical trace of the enzyme.

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FIG. 2. Part of sagittal section of adult female *Apanteles glomeratus* L. showing three mature ovarian eggs; gcd, germ-cell determinant; n, nucleus.

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DISCUSSION

The question of the origin, nature, and significance of the germ-cell determinant, especially in the unfertilized eggs of Hymenoptera, has been a subject of diverse opinions amongst the various workers. SILVESTRA (1908) called this inclusion the "nucleolo" and HEGNER (1914a, b) the "keimbahn" or "germ-cell" determinant. Both of these authors originally believed that it arose from nuclear material. It has been shown by GATENBY (1920) that the germ-cell determinant is derived from the cytoplasm of the young oöcyte. This author, who worked with more recent cytological technique, stated that the germ-cell determinant in the unfertilized egg of *Apanteles glomeratus* is formed only by albuminous material, and there is no connexion between this structure and the chromatin of the oöcyte nucleus or the follicular nuclei. HOGBEN (1920), who worked on oögenesis in the Hymenoptera, agrees with GATENBY in the origin and cytological constitution of this inclusion. The application of Feulgen's technique by MUKERJI (1930) to the oögenesis of *Apanteles* failed to reveal any relationship between the germ-cell determinant and the chromatin of the nucleus. He also mentioned that the basophilic constituents of this inclusion are not dissolved away by acid hydrolysis.

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The fact that the germ-cell determinant is a cytoplasmic inclusion provides confirmation of the view of organ-forming areas in the cytoplasm of the egg. GATENBY (1917, 1918) stated that the function of this inclusion in *Trichogramma evanescens* Westw. seems to be that of preventing the germ-cell nuclei from being exposed to the uncertain conditions existing elsewhere in the segmenting egg and embryo. He also suggests that the germ-cell determinant functions in nutrition. DU BOIS (1932) found that two of the cleavage nuclei in the egg of *Sciara coprophila* penetrate the germ-cell determinant and become differentiated as the germ cells, which apparently absorb the granular material of the disk and become greatly enlarged and altered in appearance. HEGNER (1908), by killing the posterior ends of chrysomelid beetle eggs with a hot needle, was able to get embryos without germ cells, but as a rule a part of the posterior end of the abdomen was also absent. Recent writers, as stated by JOHANNSEN and BUTT (1941), regard the germ-cell determinant merely as an indicator of the path of the germ cells from the original position at the posterior end of the egg to their definitive position in the gonad, rather than as a determinant.

It can be stated that the germ-cell determinant in most insects appears to break down into parts or granules early during the cleavage stage of the egg. Fig. 1 shows the dividing inclusion in *Brachyrhinus* (a), *Sciara* (b), *Calliphora* (c), *Litomastix* (d) (JOHANNSEN and BUTT, 1941) and *Trichogramma* (e) (GATENBY). In all these cases the resulting material of the disk is spread around the nuclei, which accordingly become germ cells. In *Apanteles glomeratus* the germ-cell determinant appears to produce small granules as a sign of its breakdown, and this usually happens very early after oviposition and during the formation of the polar bodies (Fig. 5). In the last observations it could be stated that the germ-cell determinant, where early segregation of germ cells occurs, gradually undergoes

histolysis. The cleavage nuclei that accidentally enter the resulting material will be the primordial germ cells.

YAO (1950a, b) found that the increase of alkaline phosphatase activity is almost concomitant with the actual process of histolysis during the development of *Drosophila melanogaster*. He also stated that this happens in every larval organ that is going to be destroyed. Regarding this, it could be stated that the breakdown of the germ-cell determinant in the newly laid egg of *Apanteles glomeratus* is probably due to the activity of pre-existing enzyme. The positive result of the test for alkaline phosphatase of the disk in the egg before oviposition seems to confirm this statement.

YAO (1950a, b) stated that alkaline phosphatase in embryogenesis seems to be more concerned with histodifferentiation than with chemodifferentiation. Therefore, the localization of this enzyme in the germ-cell determinant indicates that this cytoplasmic inclusion is an organ-forming area and not an indicator of the path of the primordial germ cells from the original position at the posterior pole of the egg to their definitive position in the gonad.

In the writer's opinion, it is not the germ-cell determinant but the products of its histolysis that is the factor governing the conversion of the cleavage cells into germ cells.

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THE EFFECT OF FLIGHT ON THE TASTE THRESHOLD AND CARBOHYDRATE UTILIZATION OF *PHORMIA REGINA* MEIGEN

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Abstract—The threshold of *Phormia regina* to glucose has been shown to decrease with increasing duration of flight. The rate of fall of threshold increased at high temperatures and decreased at low temperatures, and an attempt was made to relate this effect to the rate of energy expenditure by measuring the wing-beat frequency of flies at various temperatures. Wing-beat frequency measurements indicated a positive correlation with temperature, and it was concluded that threshold decrease was in some way related to energy expenditure. Estimations of carbohydrate depleted from the blood and tissues during starvation and flight indicated that blood sugar, crop sugar, and glycogen depletion *per se* were not directly related to threshold decrease. Estimations of total carbohydrate depletion during flight revealed no simple relationship between decrease in threshold and the expenditure of energy. Injection of glucose into exhausted flies enabled them to resume flight almost immediately; injections did not bring about changes in threshold. Exhausted flies which had been injected with glucose, and which had also been fed with a measured amount of sugar, appeared to utilize injected sugar first to resume flight. While injected sugar was being used, removal of sugar from the gut was retarded and the threshold remained high. It was concluded that a high threshold could be maintained, even over a period of high energy expenditure, while the gut contained a large quantity of sugar.

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INTRODUCTION

THE lowering of taste threshold to sugar solutions shown by blowflies over prolonged periods of starvation (EVANS and DETHIER, 1957) occurs much more rapidly during the performance of continuous flight. This effect has also been observed with honey-bees, and the relationship between duration of flight and lowering of taste threshold has been suggested by DETHIER (1957) in a physiological explanation of the distance component of bee dancing. Since it is probable that carbohydrate is used exclusively as the source of energy for the flight of *Diptera*, these insects provide an excellent opportunity for the investigation of any relationship between the lowering of taste threshold to sugar solutions and the depletion of carbohydrate during flight.

Evidence of the utilization of carbohydrate by *Diptera* and by bees and wasps has been presented on many occasions. WILLIAMS, BARNESS, and SAWYER (1943) estimated glycogen depletion in *Drosophila funebris* and *Lucilia sericata* by direct microchemical methods and found that glycogen both in the thorax and in the

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entire animal progressively diminished during flight. Measurements of wing-beat frequency showed that, although glycogen was decreasing, the initial rate of wing-beat was maintained until a certain critical level of glycogen was reached; after this a sharp decrease in rate occurred. It was assumed that below this level wing-beat frequency decreased with fuel depletion. BEUTLER (1937) found that the blood sugar of bees could be regulated while the honey stomach contained supplies. When these were used up, blood sugar decreased with subsequent duration of flight until a level was reached below which no further flight could be performed. Bees were unable to recover after flights to this point. This fact indicates that no other sources of supply than carbohydrate could be utilized to maintain life. The utilization of carbohydrate by *Drosophila virilis* and *Drosophila americana* in flight has been indicated by CHADWICK (1947), who measured the respiratory quotient; during flight this was found to be essentially 1·0. He also found that individuals which survived for some hours after flight, and whose carbohydrate reserves were greatly depleted, showed respiratory quotients of 0·70 or less. WIGGLESWORTH (1949) demonstrated histochemically that glycogen and fat in *Drosophila* were depleted concurrently during starvation, but that carbohydrate alone was used during flight. By feeding measured amounts of glucose to exhausted insects it was possible to determine the amount of energy used by *Drosophila* during flight. CLEMENTS (1955) showed by histochemical methods that glycogen disappeared from the fat body of mosquitoes flown to exhaustion.

During the course of such experiments, occasional observations have been made of insects which unaccountably ceased flying, either temporarily or permanently, even though their carbohydrate supplies were not exhausted (e.g. CHADWICK, 1947; SOTAVALTA, 1954); the reason for this might be attributed either directly to asynchrony of production of metabolites with demand, or indirectly to the effect of depletion or accumulation of some substance on the central nervous system. In recent years a great deal of evidence has been accumulated pointing to the occurrence of metabolic pathways in insects similar in general to those known to exist in other organisms; especially pertinent are the works of BARRON and TAHMISIAN (1948), SPIRTES (1951), CHEFURKA (1954, 1955), and SACKTOR (1955). No attempt has been made here to investigate the relationship between threshold changes and the presence of metabolic intermediates, but experiments in this direction might well give more definite information on the regulatory mechanism of threshold.

The relationship between taste threshold and duration of flight of bees has been described by DETHIER (1957) and by EVANS and DETHIER (1957). Bees fed to repletion on 2 M sucrose showed a drop in threshold to sucrose from 2 M to 0·125 M after a period of about 1½ hr of continuous flight; at the end of this time the honey stomach (crop) was almost empty. Starvation of bees fed on 2 M sucrose also produced a drop in taste threshold, but the time required to reach a minimum value was greater than 60 hr. EVANS and DETHIER (1957), in experiments to discover the regulatory mechanism of taste threshold in *Phormia regina*, investigated the blood sugar and crop sugar content at various intervals during

starvation. They found that the chief blood sugar was trehalose and that, after the insects had been fed to repletion on 2 M glucose, the trehalose value in the blood reached a peak level 10 hr later. This level was maintained up to 50 hr, after which there was a steady decline until the lowest value was reached at approximately 80 hr after feeding. Blood glucose was rapidly reduced to a low level which was steadily maintained. Since threshold measurements for glucose showed a progressive decrease over the entire period, it was concluded that blood sugar level *per se* was not the regulating factor. This conclusion was supported by the fact that sugars, injected even at concentrations far in excess of those normally maintained in the fly, did not elevate the taste threshold. Measurements of crop contents at intervals after feeding sugar solution showed a progressive decrease in volume although the concentration of sugar was maintained constant. The suggestion that crop content could be a regulatory factor was discouraged by experiments in which the crop was ligatured before and after feeding; in neither case was there any significant effect on threshold.

The present work is an attempt to gain more information on the factors concerned with threshold regulation. The threshold of *Phormia regina* Meigen has been measured at certain intervals during continuous flight to exhaustion; the effect of temperature on the rate of decrease of threshold has been investigated to determine the relation between energy expenditure, measured by increase or decrease of wing-beat frequency, and changes in taste sensitivity. The depletion of blood and tissue carbohydrates has been estimated after similar intervals of flight to find out whether a decrease of carbohydrate from any one source could be related to decrease in taste threshold. In the course of these experiments some additional information has been gained on the rate of synthesis of glycogen by flies fed on measured amounts of glucose and upon its subsequent depletion during starvation.

MATERIALS AND METHODS

Three-day-old flies maintained on 0·1 M sucrose solution were isolated in a jar and fed on 1·0 M glucose, sprayed on to a filter paper, on the day before the experiment. The paper was removed after feeding, and 24 hr later individual flies were fed a measured amount of glucose and flown for the time required. Thresholds were obtained by touching the tarsi on to shallow solutions of sugar contained in Petri dishes. The normal procedure for flight experiments was as follows: flies were fed 1·8 mg of 1 M glucose and allowed to rest for 1 hr, and at the end of this time they were offered water and the threshold to glucose was obtained; this is referred to as the initial threshold. Whenever the threshold was measured, flies were first offered water and allowed to drink as much as they would; little was taken during flight even at high temperatures. Flights at 39° C were carried out in a room constantly maintained at this temperature and at high humidity; at 33° C, in a small room heated by an electric heater; at 21–22° C, in a room cooled by an air conditioner. Wing-beat frequencies were measured stroboscopically with a General Radio Company Strobotac, type 631-B.

In these experiments *Phormia* could not be induced to fly continuously in a fixed position on a flight mill, even with the assistance of visual stimuli, air currents, and sudden removal of the substratum from the tarsi. Successful results were obtained with a method similar to that described by HOCKING (1953) for *Tabanus affinis:* a light thread was waxed on to the mesothorax, and the flies were suspended above a turntable angled so that the tarsi could just make contact with it in one position on the circumference. When the turntable began to revolve, the flies were forced into flight as the length of thread reached its full extent; each time they attempted to land, the revolution of the wheel carried them into flight again, and the combination of stimuli produced by this method maintained flight to exhaustion. Exhaustion was assumed when successive flights were reduced to less than 10 sec intervals. Flight could always be oriented towards a light source or a white background, which helped to prevent the tendency to fly back on to the thread itself. The best results were obtained when a number of flies (six) were flown simultaneously; the flight sounds of neighbouring insects possibly acted as a stimulus to reluctant fliers. Even under the most favourable conditions there were always some flies which consistently refused to fly and others which performed intermittently; data have been obtained only from those which could be stimulated to fly continuously for the required length of time.

Carbohydrate contents were estimated in the following way: a sample of blood was taken by removing the front legs and compressing the thorax so that a drop was forced out; this was taken up into a graduated micropipette and expelled into 1 ml of 5% trichloroacetic acid (EVANS and DETHIER, 1957). Crops were removed through an opening in the first abdominal segment and crushed in 95% methanol. The remainder of the fly was ground up in 95% methanol and centrifuged. The supernatant and two successive washings with methanol were removed and evaporated under vacuum. The carbohydrate fraction obtained was redissolved in water to the required dilution. This fraction represented sugar present in the midgut, together with any other alcohol soluble carbohydrate present after the crop and 3–4 μl of blood had been removed. Since the sugar in the midgut formed a large proportion of this fraction, the whole fraction is referred to as midgut sugar in the following account. The residue from the methanol extraction was boiled for 15 min in 1 ml of 30% potassium hydroxide, cooled and centrifuged, and the supernatant removed. The residue was washed twice with KOH. The glycogen extracted by this procedure was precipitated out by adding ethanol in the proportion of 1·5 vol of 95% ethanol to 1 vol of KOH. Precipitation was aided by cooling to 3° C. The precipitate containing glycogen was centrifuged down and the supernatant fluid was drained off; glycogen was redissolved in water (after the method of SEIFTER *et al.*, 1950).

Dilutions of all carbohydrates obtained by these procedures were estimated by the method of DIMLER *et al.* (1952) which employs Anthrone in sulphuric acid and gives accurate determinations of reducing and non-reducing sugars. Glycogen was calculated using the factor determined by MORRIS (1948) for conversion of glucose to glycogen. Unless otherwise stated, the values for carbohydrates are

total carbohydrate values, which in the case of blood represent the sum of trehalose and glucose present. Separations of sugars were made chromatographically according to the methods described by EVANS and DETHIER (1957). Eluted sugars were estimated by the Anthrone method or by the method of PARK and JOHNSON (1949) for small quantities of reducing sugars.

Injections were performed using the micro-injection apparatus of BUCK (1949) with a 27 gauge, $\frac{1}{2}$ in. hypodermic needle. Flies were not apparently damaged by injection and normal flight could be resumed afterwards.

RESULTS AND DISCUSSION

The effect of duration of flight on threshold

At room temperature, 25° C, threshold falls to less than 10% of the initial value after 110 min of flight. With control flies mounted on waxed sticks and fed similarly, no fall in threshold occurs over this time period. During flight, threshold falls exponentially with time (Fig. 1), and even after 3 min of activity a decrease to 81% of the initial value occurs.

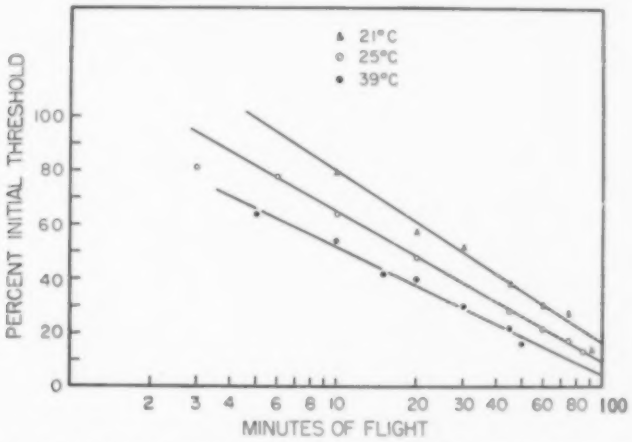

FIG. 1. Relation of threshold decrease to time of flight at three temperatures.

Because of the variation in initial threshold, even between carefully standardized flies, and because every fly did not necessarily respond each time the threshold was tested, each measurement has been calculated individually as per cent initial threshold.

At 39° C the decrease in threshold occurs more rapidly and at 21° C more slowly than at room temperature; in both cases, to allow equilibrium to be reached, the flies and test solutions were kept at the higher and lower temperatures for 1 hr before flight was begun. At 39° C flies must have been very close to their thermal death point; however, continuous flight was maintained in the manner

described, and the fact that the flies were able to perform at this temperature was probably due to the high humidity maintained in the room.

To determine whether the more rapid decrease of threshold at higher temperatures could be related to greater energy expenditure, wing-beat frequencies were measured on a few flies at different temperatures. At 25° C the average number of cycles per minute during the first 10 min of flight was found to be 12,000 c/min, at 33° C, 13,200 c/min and at 22° C, 9540 c/min. There is, therefore, an increase in the rate of flight at high temperature and a decrease at a low temperature. The relationship could not be accurately determined on the small number of flies used for these observations; however, it is indicated that with *Phormia* there is a positive correlation between wing-beat frequency and temperature, over the range tested, similar to that shown by CHADWICK (1939) with *Drosophila*.

The fact that threshold decrease and wing-beat frequency are both accelerated at high temperatures suggests that threshold is in some way related to energy expenditure. It was hoped that an investigation of carbohydrate depletion during flight might give some direct indication of the factors involved.

Relation between threshold and carbohydrate depletion

To establish the normal glycogen and midgut sugar contents of *Phormia regina* under the conditions of nutrition maintained for these experiments, certain basic estimations were made on flies selected and treated as for flight experiments, but mounted on waxed sticks. These flies were starved for 48 hr and observations made at intervals; measurements of crop glucose and total carbohydrate value of blood were included to give a complete set of data on one group of flies. The values obtained are given in Table 1, and those estimated at 1–48 hr after feeding

TABLE 1—CARBOHYDRATE ESTIMATIONS IN FLIES AFTER FEEDING (85 FLIES)

Time after feeding (hr)	Total carbohydrate value of blood (g/l.)	% initial value	Glycogen (μg/fly)	% initial value	Crop sugar (μg/fly)	% initial value	Midgut sugar (μg/fly)	% initial value
Immediately (initial value)	12·3	100	189·1	100	2790·0	100	118·4	100
1	15·1	123	433·8	229	2210·0	79	169·0	143
3	16·7	135	474·8	256	1408·0	54	251·3	212
6	24·3	197	555·5	293	1038·0	37	159·9	135
12	28·4	230	538·7	307	689·8	25	132·3	112
24	15·9	129	790·5	417	307·0	20	131·0	111
30	8·5	69	442·6	234	232·5	8	148·2	125
36	4·6	37	420·4	227	404·0	15	77·1	65
48	8·5	69	203·2	107	71·9	3	80·3	68

are calculated as per cent of the amounts estimated immediately after feeding. Changes in per cent initial value with time of starvation are shown in Fig. 2.

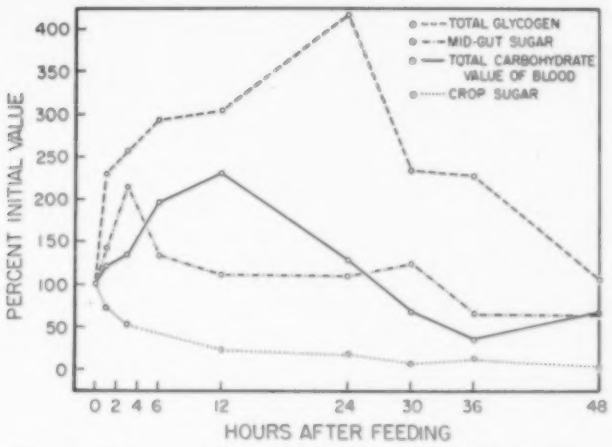

FIG. 2. The course of carbohydrate depletion during starvation.

Glycogen reserves increased rapidly after feeding. The increase of sugar in the midgut was presumed to be due to the fairly rapid emptying of the crop during the first 3 hr; the gut sugar was thereafter depleted and its decrease was reflected by a rise in blood sugar. The rise in blood sugar appeared to be maintained until supplies from the crop *via* the gut could no longer keep pace with withdrawal of sugar from the blood, and at this point blood sugar began to fall. Glycogen synthesis continued until blood sugar was reduced to approximately 16·0 g/l., after this, glycogen was depleted with increasing time of starvation. The tarsal threshold of these flies, to glucose, had decreased to 85% after 6 hr, to 58% after 12 hr, and to 30% of the maximum after 24 hr.

These results confirm the fact that threshold at first increases with blood sugar and that only at a critical time, when the crop and other sources are unable to maintain the level, does threshold fall with blood sugar. Blood sugar *per se* cannot, therefore, be considered as a regulatory factor. The results also show that glycogen must be eliminated as a controlling factor since it is still increasing at a time when threshold has fallen to about 30% of its maximum.

During flight experiments, considerable variation in carbohydrate content was found between control flies from different groups. The measurements from flown flies have been calculated as percentages of those obtained from controls, from the same group, mounted on sticks but otherwise treated identically (Table 2, Fig. 3). The most striking difference between starved and flown flies is in the rapid disappearance of glycogen in the latter, despite the fact that the crops contained glucose which could become almost instantaneously available (WIGGLESWORTH, 1949) (see also below). Glycogen had decreased to 76% of the control value after

15 min of flight, and crop sugar to 83·4% of the controls. Sugar from the crop was presumably being measured in the blood and midgut since both these values increased during the first 15 min. A total of 204 μg of carbohydrate disappeared from these flies during this period, all of which could not be accounted for by

TABLE 2—CARBOHYDRATE ESTIMATIONS IN FLIES AFTER FLIGHT EXPRESSED AS GLUCOSE

Duration of flight (min)	No. of flies (control and flown)	Total carbohydrate value of blood (g/l.)			Glycogen (μg/fly)			Crop sugar (μg/fly)			Mid-gut sugar (μg/fly)			Total carbohydrate depletion (μg/fly)	Rate of carbohydrate utilization (μg/min)
		Control	Flown	Flown as % control	Control	Flown	Flown as % control	Control	Flown	Flown as % control	Control	Flown	Flown as % control		
15	22	17·0	23·7	139	604·4	464·5	76	980·3	740·3	83	158·9	294·5	123	205	13·6
30	17	27·1	25·3	93	537·9	300·9	56	1269·5	930·5	73	196·9	222·7	113	561	18·7
45	22	19·2	18·7	97	709·3	230	32	883·3	559·8	63	185·4	179·8	97	813	18·0
60	21	17·0	16·7	98	373·6	103	28	1195·0	612·7	51	125·3	96·9	83	882	14·7
180	10	26·6	12·4	46	552	30·9	5	1151·0	206·0	18	220·6	46·0	21	1·724	9·4

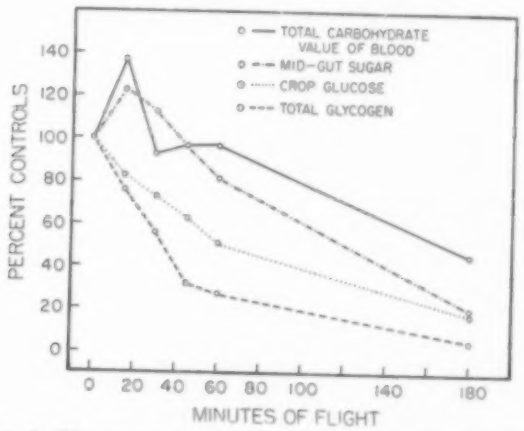

FIG. 3. The course of carbohydrate depletion during flight.

glycogen depletion. Threshold had decreased to 55% of its initial value after 15 min. Measurements of carbohydrate at subsequent intervals to 180 min of flight showed a fairly steady decrease of glycogen, crop sugar, and midgut sugar; blood sugar decreased sharply during the period 15–30 min after the commencement of flight and was then maintained at a certain level from 30–60 min. This steady level may have continued over a longer period, but no estimations were made between 60 and 180 min. Threshold decreased fairly rapidly up to 60 min and more slowly as the lowest concentration at which flies can detect glucose was approached. Threshold did not change when flies were allowed to rest for 2–3 hr after flight had ceased.

On the basis of these and previous experiments it is possible to eliminate blood sugar, crop sugar, and glycogen as factors immediately affecting threshold regulation. The effect of sugar in the midgut remains to be considered. Certain ways in which the midgut could be involved in threshold regulation have already been suggested; these include regulation through receptors situated in this region which could be affected by concentration of sugar or by distension. It is also possible that the rate of absorption of sugar, i.e. the concentration of sugar within certain cells of the midgut, could either directly or indirectly affect threshold. The estimations of midgut sugar during flight showed that this was at first increased while at the same time threshold was falling; however, since the volume of fluid in the gut was not estimated, it is still not known how the concentration varied.

With the possibility in mind, of regulation of threshold by some effect in the midgut, it was of interest to observe the effect of injecting exhausted flies with a quantity of glucose, calculated to maintain flights of a definite duration, but which, it had been found, did not affect threshold, and at the same time feeding them with a non-utilizable sugar. Since the fed sugar could not be metabolized it was possible to observe its disappearance from the gut and its appearance in the blood. Elimination of carbohydrate by excretion during flight could be followed by examination of excretory drops collected on paper chromatograms. Thresholds were tested after injection, after feeding, and after flight.

A group of flies were flown to exhaustion, at which time they responded to tarsal contact with 0·06 M glucose. They were then injected with an amount of glucose calculated to maintain flight for approximately 40 min; the injection did not raise the threshold. Immediately afterwards they were fed with 1·3 mg of fucose which raised the glucose threshold to 1 M. The flies were then flown until they were again exhausted (within 38–40 min). The threshold at exhaustion was not significantly lower than at the beginning of flight. Estimations of crop fucose showed that this had decreased very little, to 95% of the amount fed; fucose in the blood was measured at 15 g/l.; the amount of glucose was negligible; sugar in the midgut varied from 250–283 μg. Excretory drops collected during flight were not found to contain carbohydrate. It was concluded that injected sugar was used for flight; when this was exhausted no further flight could take place. Very little fucose had been removed from the gut and the threshold was high, even after a flight period which would normally bring about a decrease to 30% of the initial value. This was then a case in which energy had been expended to a point at which flight could no longer be maintained and yet threshold remained high, and it is interesting to consider this from a behaviour point of view.

To compare the effect on threshold of injecting and feeding exhausted flies with a utilizable sugar, a second group of flies were injected with a similar quantity of glucose to that used above and fed with 1·2 mg of glucose. Threshold was measured at the commencement of flight, after 30 min, and at subsequent 15 min intervals during flight to exhaustion. The results are summarized in Table 3. After 45 min of flight the threshold had decreased by only 2%, whereas in flies fed, but not injected, the threshold had decreased to 30% in this time. Crop glucose

had only decreased to 90% of the amount fed after 45 min and blood carbohydrate was measured at 21 g/l.; sugar in the midgut varied from 200 to 300 μg. It was again concluded that until this time injected glucose had been used to maintain flight. Subsequent measurements of threshold showed that this decreased in the manner shown in earlier experiments.

TABLE 3—THE EFFECT OF GLUCOSE INJECTION FOLLOWED BY GLUCOSE FEEDING ON THE TARSAL THRESHOLD OF *Phormia regina*

Time after feeding and injection (min)	Mean tarsal threshold to glucose (Molar)	% initial threshold
Immediately	1·08	100·0
30	1·08	100·0
45	0·98	90·7
60	0·54	46·5
75	0·46	42·5
90	0·34	31·2
105	0·21	19·6
120	0·23	20·9
135	0·12	11·4

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It is apparent from these results that the threshold remained high while the midgut contained sugar in large quantity, but when the sugar was withdrawn from the gut, threshold decreased. Clearly, vigorous energy expenditure can occur without a decrease in threshold; however, it must be kept in mind that these conditions were quite abnormal.

In view of this finding an attempt was made to relate the total energy expenditure, expressed as total carbohydrate utilization per unit time (Table 2), under normal conditions of feeding, to decrease in threshold over similar periods of time. From calculations of total carbohydrate utilized per minute for each 15 min period of flight, it appeared that energy expenditure took place at a fairly constant rate for a period of at least 60 min. When wing-beat frequency was measured over long periods, it was found to remain fairly constant over similar periods of time. Threshold, however, showed a sharp decrease very soon after flight had begun, and continued to decrease exponentially with time. No simple relationship is demonstrable between threshold and energy expenditure.

Some aspects of flight metabolism of Phormia regina

From the data obtained during the course of these experiments it has been possible to make certain calculations of carbohydrate utilization and energy expenditure by *Phormia* in flight. The average figure obtained for carbohydrate utilization, expressed as glycogen, was found to be 14·79 μg/min. This figure was obtained by calculating the total carbohydrates depleted from the blood [assuming 6 μl/fly (BEARD, 1953)] and tissues, from the figures given in Table 2. Total

carbohydrate depletion has been calculated by subtracting the total carbohydrate of flown flies from that of control flies at 15 min intervals from 0 to 60 min. This procedure gave the total depletion (expressed as μg glucose utilized per min) per 15 min; glycogen has been calculated as glucose $\times$ 0·91 (MORRIS, 1948).

The average wet weight of *Phormia regina* was found to be 25 mg/fly, and the wet weight of the flight muscles [calculated by subtraction of the weight of the dry thorax, after treatment with KOH, from the weight of the live thorax (CHADWICK and GILMOUR, 1940)] was 10 mg/fly. The average wing-beat frequency at room temperature was 12,000 strokes/min. With these measurements of carbohydrate utilization, weight, and wing-beat frequency it has been possible to calculate the oxygen consumption and total energy expenditure of *Phormia;* to give figures comparable with those obtained for other insects (CHADWICK, 1953; HOCKING, 1953; SOTAVALTA, 1954); these figures are shown in Table 4.

TABLE 4—GLYCOGEN UTILIZATION, OXYGEN CONSUMPTION AND TOTAL ENERGY EXPENDITURE OF *Phormia regina*

	Glycogen utilization	Oxygen consumption at 25° C	Total energy expenditure
Per hour	35·5 mg/g	31·72 cm^3/g	152 cal/g
Per wing-stroke	$1{\cdot}24 \times 10^{-7}$ g/g muscle	$1{\cdot}11 \times 10^{-4}$ cm^3/g muscle	$0{\cdot}521 \times 10^{-3}$ cal/g muscle

Injections—To determine, by a direct method, the amount of glucose required to maintain flight for a stated time, flies were flown to exhaustion and injected with a measured amount of glucose. They were then flown again, and the time of flight to a second state of exhaustion was recorded. The amount of glucose utilized per hour of flight was found to be 918 μg/fly or 36·7 μg glycogen/g/hr. This is in good agreement with the figure for glycogen utilization shown in Table 4. It should be mentioned that when glucose was fed to exhausted flies they were able to resume flight within 30 sec (cf. WIGGLESWORTH, 1949).

Glycogen reserves—The investigation of glycogen content in *Phormia*, made in the earlier part of this work, indicated that the flight muscles contained only about 35% of the total glycogen content of a fly; the remainder was contained in the abdomen. The fat body of *Phormia* is very diffuse, but large numbers of cells are packed laterally in the abdomen; it is probable that these contain a large part of the glycogen reserve.

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THE OCCURRENCE OF *PARA*-QUINONES IN SOME ARTHROPODS, WITH EMPHASIS ON THE QUINONE-SECRETING TRACHEAL GLANDS OF *DIPLOPTERA PUNCTATA* (BLATTARIA)

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Abstract—The tracheae leading to the second abdominal spiracles of nymphs and adults of *Diploptera punctata* are odoriferous glands which are probably used as a means of defence against predators. By infra-red and mass spectrographic analyses the odorous secretion was identified as a mixture of 2-ethyl-1,4-benzoquinone; 2-methyl-1,4-benzoquinone; and *para*-benzoquinone. The morphology, histology, and histochemistry of the quinone-secreting glands are described. A polyphenol oxidase and a β-glucosidase were demonstrated in the gland cells but no β-glucoside was isolated. Tracheal glands associated with abdominal spiracles are noted in several other genera of Blattaria but no quinones appear to be produced by them. Some other arthropods known to secrete quinones are listed. The secretion from the abdominal and thoracic odoriferous glands of the tenebrionid beetle *Diaperis maculata* was identified as a mixture of 2-ethyl-1,4-benzoquinone and 2-methyl-1,4-benzoquinone.

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INTRODUCTION

QUINONES, aside from the *ortho*-quinones which are involved in tanning of the cuticle, have been found in a number of arthropods. Flour beetles (e.g. *Tribolium castaneum* Herbst) secrete a mixture of 2-ethyl-1,4-benzoquinone; 2-methyl-1,4-benzoquinone (ALEXANDER and BARTON, 1943; LOCONTI and ROTH, 1953) and traces of 2-methoxy-1,4-benzoquinone (LOCONTI and ROTH, 1953); ethylquinone comprises about 80 to 90 per cent of the secretion and the methylquinone about 10 to 20 per cent. The histology of the quinone-secreting glands of *Tribolium confusum* J. du V. has been described by ROTH (1943) and of *Tribolium destructor* Uyttenb. by PALM (1946). BÉHAL and PHISALIX (1900) found *para*-benzoquinone ("la quinone") in the myriapod *Julus terrestris*. BARBIER and LEDERER (1957) have identified quinones in two other species of millipedes; *Spirostreptus castaneus* Attems contains *para*-benzoquinone, and *Spirostreptus virgator* Sylvestri emits a mixture of methylquinone and two other unidentified quinone-like substances. VERHOEFF (in BURTT, 1947) stated that the most frequent hues of exudates of millipedes are yellow–brown or brown, but red, lemon–yellow, white, or colourless secretions also occur. Most of the *para*-quinones are yellow, whereas the majority of *ortho*-quinones are orange or red (FIESER and FIESER, 1944).

Recently, di- and trisubstituted quinones have been found in a South American arachnid (Gonyleptidae) (ESTABLE *et al.*, 1955; FIESER and ARDAO, 1956). These

workers found 2,3-dimethyl-1,4-quinone (the major quinone of the mixture); 2,5-dimethyl-1,4-quinone; and 2,3,5,trimethyl-1,4-quinone. Unidentified quinones have been found in the tenebrionid beetles *Latheticus oryzae* Waterh. and *Tribolium destructor* (LOCONTI and ROTH, 1953). We have also found (unpublished data) quinones in two other adult tenebrionids, *Scotobates calcaratus* (Fabricius) and *Uloma impressa* Melsheimer. These beetles were collected in rotting logs, but because of insufficient specimens the types of quinones could not be determined.

The only reference to the secretion of *Diploptera punctata* (Eschscholtz) [= *Diploptera dytiscoides* (Serville), (PRINCIS, 1950)] is apparently by WILLIAMS (1944), who wrote that this cockroach has a "buggy odor".

In the present study, the quinones of the tenebrionid beetle *Diaperis maculata* Olivier and the cockroach *Diploptera punctata* are identified and the tracheal glands of *D. punctata*, which secrete the quinones, are described.

MATERIALS AND METHODS

Diaperis maculata were collected from the fungus *Polyporus betulinus* Bull. ex Fries, which grows on birch trees. *Diploptera punctata* were reared in the laboratory on Purina Dog Chow Checkers.

The odorous secretions were identified by means of infra-red analysis and mass spectrometry. The method of mass analysis was similar to that recently described for the identification of the odorous secretion of *Eurycotis floridana* (ROTH *et al.*, 1956). For infra-red analysis, the tracheal glands of *D. punctata* were dissected from the insects and placed in the solvent. The secretion of *D. maculata* was collected in a dry ice and acetone trap by passing air over 200 adult beetles that were alternately cooled and warmed (ROTH and HOWLAND, 1941). For purposes of comparison infra-red spectra were made of several known synthetic quinones.

The various techniques used in the histological, histochemical, and enzymological portions of this study are described in the sections of the paper in which they occur.

OBSERVATIONS

The odorous secretion of Diploptera. If several specimens of this cockroach are agitated in a closed tube, a distinctive odour similar to the quinones of the flour beetles *Tribolium* spp. is detected. When pressure is applied to the abdomen of an adult of *Diploptera* held near one's nose, the characteristic pungent odour is evident.

The similarity between the odorous secretion of *D. punctata* and that of the quinones from *Tribolium* is further indicated by the following observations. If the cockroach is inactivated by cooling with ice or solidified carbon dioxide, the secretion may ooze from the second abdominal spiracles and solidify into yellow crystals on the abdomen. Sometimes these yellow crystals are seen on *D. punctata* anaesthetized with carbon dioxide at room temperature. The yellow secretion can be collected in a dry ice and acetone trap by passing air over a large number of the

insects which are alternately cooled and warmed. When *D. punctata* is dropped into concentrated sulphuric acid, the insects emit the secretion which becomes dark red. When the insect is dropped into 10 per cent potassium hydroxide, the emitted secretion becomes green; if a drop of potassium hydroxide is placed over the second abdominal spiracle the hydroxide quickly turns green. The yellow crystals give a blue–black colour with acidulated potassium iodide starch paper and also turn crystals of hydroquinone blue. All of these reactions indicate that the secretion of *D. punctata* is related chemically to that of *Tribolium* (ROTH and HOWLAND, 1941; ROTH, 1943; ALEXANDER and BARTON, 1943; LOCONTI and ROTH, 1953).

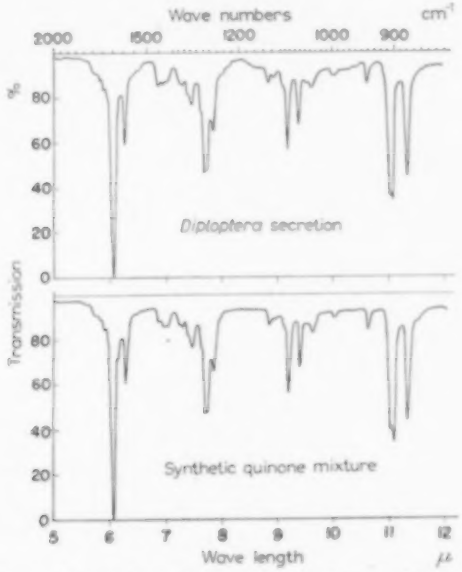


FIG. 1. Comparison of the infra-red spectra of a mixture of known quinones and the secretion of *Diploptera*. The known quinone mixture consisted of 3·6 mg of 2-methyl-1,4-benzoquinone, 2·6 mg of 2-ethyl-1,4-benzoquinone, and 1·4 mg of *para*-benzoquinone in 1 cm^3 of carbon tetrachloride. The material from *Diploptera* was obtained from 50 adult females (100 glands). Baird spectrograph with NaCl prism; cell thickness 0·418 mm.

The distinctive infra-red spectra of several *para*-benzoquinones were studied by YATES *et al.* (1956). Analysis of the infra-red spectra of the natural product and those of the synthetic quinones showed that the *Diploptera* secretion consisted of a mixture of 2-ethyl-1,4-benzoquinone; 2-methyl-1,4-benzoquinone; and *para*-benzoquinone. A synthetic mixture of these three quinones gave a spectrum which was essentially identical to that obtained from the *Diploptera* secretion (Fig. 1). An estimation of the relative proportions of the quinones in *Diploptera* can be obtained from the known mixture. The synthetic mixture (Fig. 1) consisted of about 47 per cent methylquinone, 34 per cent ethylquinone, and 18 per cent *para*-benzoquinone. During the process of removing the glands, some of the quinones may

have been lost, by volatilization or breaking of the sac, so that the percentages indicated by the synthetic mixture may not indicate the true proportions in the insects. However, several samples using different numbers of glands were analysed and in all instances the relative concentrations were methylquinone > ethylquinone > benzoquinone. Infra-red analysis of the secretion from 80 adult males (160 glands) showed the presence of the three quinones found in the females and also that the order of concentration of the three compounds was the same; however, the mixture from the males contained a slightly higher percentage of methylquinone than that found in the females. Analysis of the secretion from 130 (260 glands) last and next-to-last instar nymphs of both sexes showed the presence of the three quinones, but in the following relative concentrations: ethylquinone > methylquinone > benzoquinone; in addition, the infra-red spectrum of the nymphal secretion contained an absorption peak at about 5·75 μ, indicating an additional substance not present in the adult secretion.

TABLE 1—MASS SPECTROMETRIC ANALYSIS OF THE ODOROUS SECRETION OF *Diploptera punctata*

Note: The concentration in mole % in the vapour phase for the three quinones was ethylquinone 59·2, methylquinone 32·5, and benzoquinone 8·3. The glands from 20 adult females were used for this data. Italicized figures = parent mass.

M/e	Actual divisions of spectrum	Ethyl-quinone	Methyl-quinone	*Para*-benzo-quinone	Residual
105	3·0	2·0	0·4		+0·6
106	18·2	17·4	0·3		+0·5
107	215·0	213·4	1·0		+0·6
108	1335·0	688·3	0·3	*646·4*	0
109	98·0	54·1		43·4	+0·5
110	21·0	9·6		10·7	+0·7
111	2·0	0·5		0·7	+0·8
118	3·0	3·4			−0·4
119	1·7	2·1			−0·4
120	0·9	0·5	0·2		+0·2
121	32·7	30·2	2·2		+0·3
122	885·0	4·6	*880·4*		0
123	88·5	17·3	70·4		+0·8
124	22·2	1·3	20·3		+0·6
125	1·8		1·4		+0·4
134	3·2	3·5			−0·5
135	8·8	9·4			−0·6
136	392·0	*392·0*			0
137	35·2	35·3			−0·1
138	12·0	12·2			−0·2

Mass spectrometric analysis (Table 1) of the glands from adults of *Diploptera* confirmed the identification of the three quinones identified by infra-red analysis.

The odorous secretion of Diaperis. The odoriferous glands of *Diaperis* consist of glandular cells associated with four large reservoirs, two in the abdomen and two in the thorax (ROTH, 1945).

The infra-red spectrum of crystalline material showed that the quinones of *Diaperis* consisted of a mixture of ethylquinone and methylquinone. The infra-red spectrum of the *Diaperis* quinones is compared with that of *Tribolium* in Fig. 2, a and c. These spectra are essentially similar except for the relative proportions

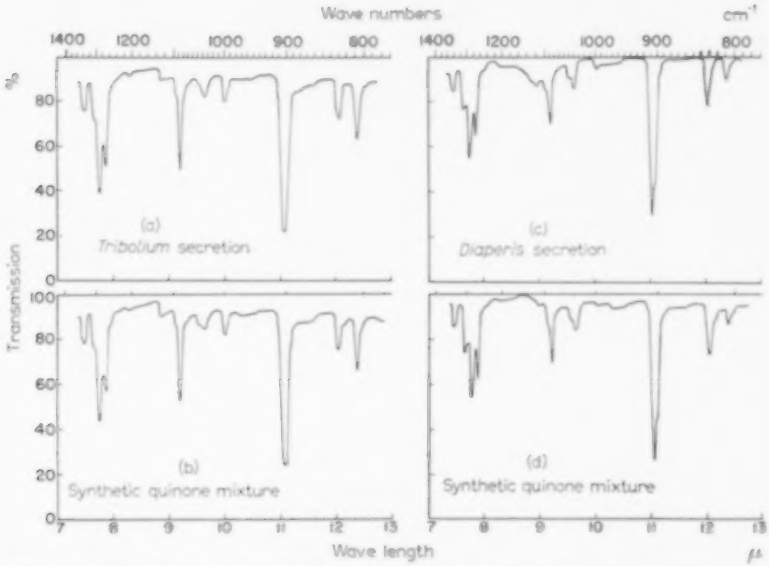


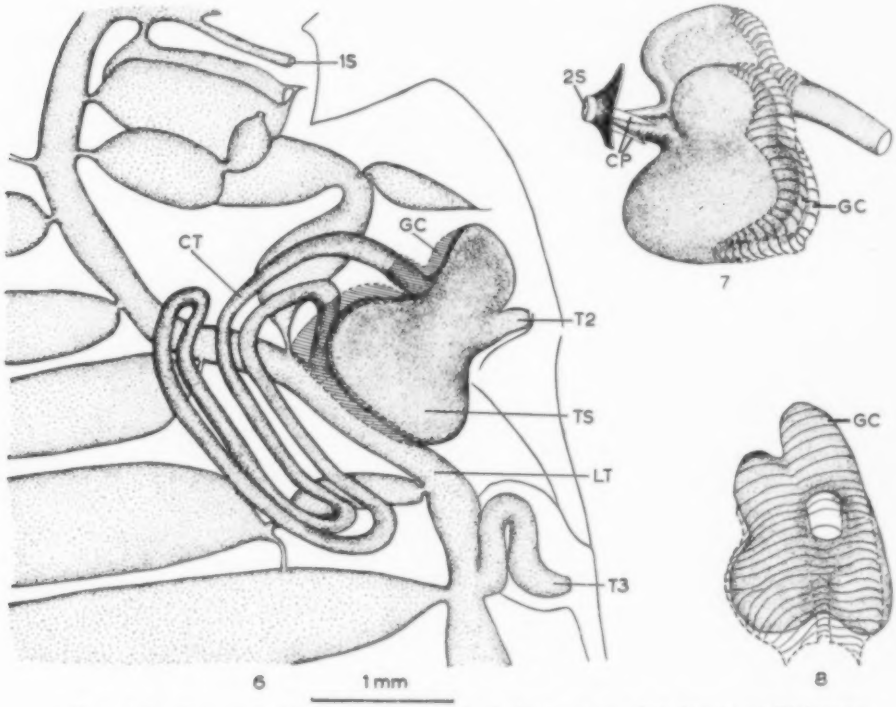
FIG. 2. Comparison of the infra-red spectra of mixtures of known quinones, and the secretions obtained from *Tribolium* and *Diaperis*. The absorption bands in the lower wavelength regions were similar for the various synthetic mixtures and natural occurring compounds. Baird spectrograph with NaCl prism; cell thickness 0·418 mm.

(a) Crystals from *Tribolium castaneum*, sublimed from *iso*-pentane under vacuum; dissolved in CS_2. (b) Synthetic quinone mixture consisting of 5 mg ethylquinone and 4·5 mg methylquinone per cm^3 of CS_2. (c) Crystals from *Diaperis maculata*, sublimed from *iso*-pentane under vacuum; dissolved in CS_2. (d) Synthetic quinone mixture consisting of 6·9 mg ethylquinone and 2·1 mg methylquinone per cm^3 of CS_2.

of the ethylquinone and methylquinone, as can be seen by a comparison of the natural products with the synthetic mixtures; a smaller proportion of methyl- to ethylquinone is found in *Diaperis* (cf. Fig. 2, (a) and (b), and (c) and (d)). Mass spectrometric analysis of the *Diaperis* secretion confirmed the infra-red findings. Although not shown in Fig. 2, infra-red spectra of the crude extracts of the quinones from *Tribolium* and *Diaperis* sometimes had two strong absorption bands between 3·4 and 3·6 μ. *Tribolium* quinones are present in an oily compound in the reservoirs of the odoriferous glands (LOCONTI and ROTH, 1953). LOCONTI (personal communication) has tentatively suggested that this oil is a mono unsaturated hydrocarbon of molecular weight 179 (Rast method). A dilute sample of the *Tribolium* oil gave a spectrum in which only the CH bands (absorption peaks at 3·4–3·6 μ) were prominent. It is likely that the strong absorption peaks

in the 3·4–3·6 μ region of the spectra of crude extracts of the secretions of *Diaperis* and *Tribolium* are due to an oil in which the quinones are dissolved.

The odoriferous glands of Diploptera. The sac-shaped swellings of the tracheae leading to the second abdominal spiracles of *D. punctata* are visible through the tergite of a freshly moulted adult (Figs. 3 and 4). The sacs or reservoirs are a golden brown colour while the cuticle is still untanned. A long, narrow tracheal tube, shown extended in Fig. 5, connects the sac with the lateral tracheal trunk. Fig. 6 shows a dorsal view of the tracheal swelling in relation to the main dorsal tracheae. The long, narrow trachea is normally coiled and enters the tracheal



FIGS. 6–8. Camera lucida drawings of tracheal glands of an adult female of *Diploptera punctata*. (CP = conical processes; CT = coiled trachea connecting the sac with the lateral tracheal trunk; GC = glandular cells; LT = lateral longitudinal tracheal trunk; 1S = first abdominal spiracle; 2S = second abdominal spiracle; T2 = trachea to second abdominal spiracle; T3 = trachea to third abdominal spiracle.)

FIG. 6. Dorsal view of a right gland in situ. FIG. 7. Ventral view of a right gland. FIG. 8. Medio-lateral view of a right gland.

trunk between the lobes of the reservoir, where it is surrounded by gland cells. In ventral view (Fig. 7) or medio-lateral view (Fig. 8), the lobed structure of the sac is evident. The glandular cells are found mostly on the medio-lateral side of the sac (Figs. 6–8, GC). However, some gland cells occur along the narrow coiled

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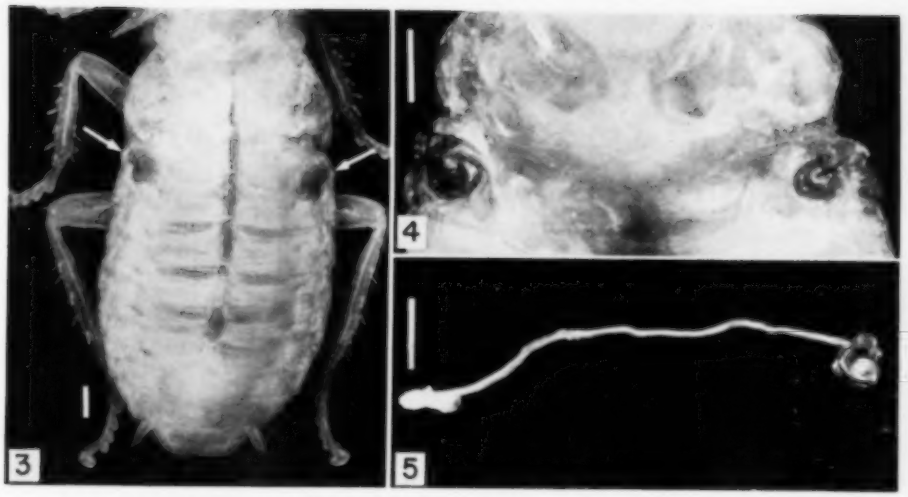
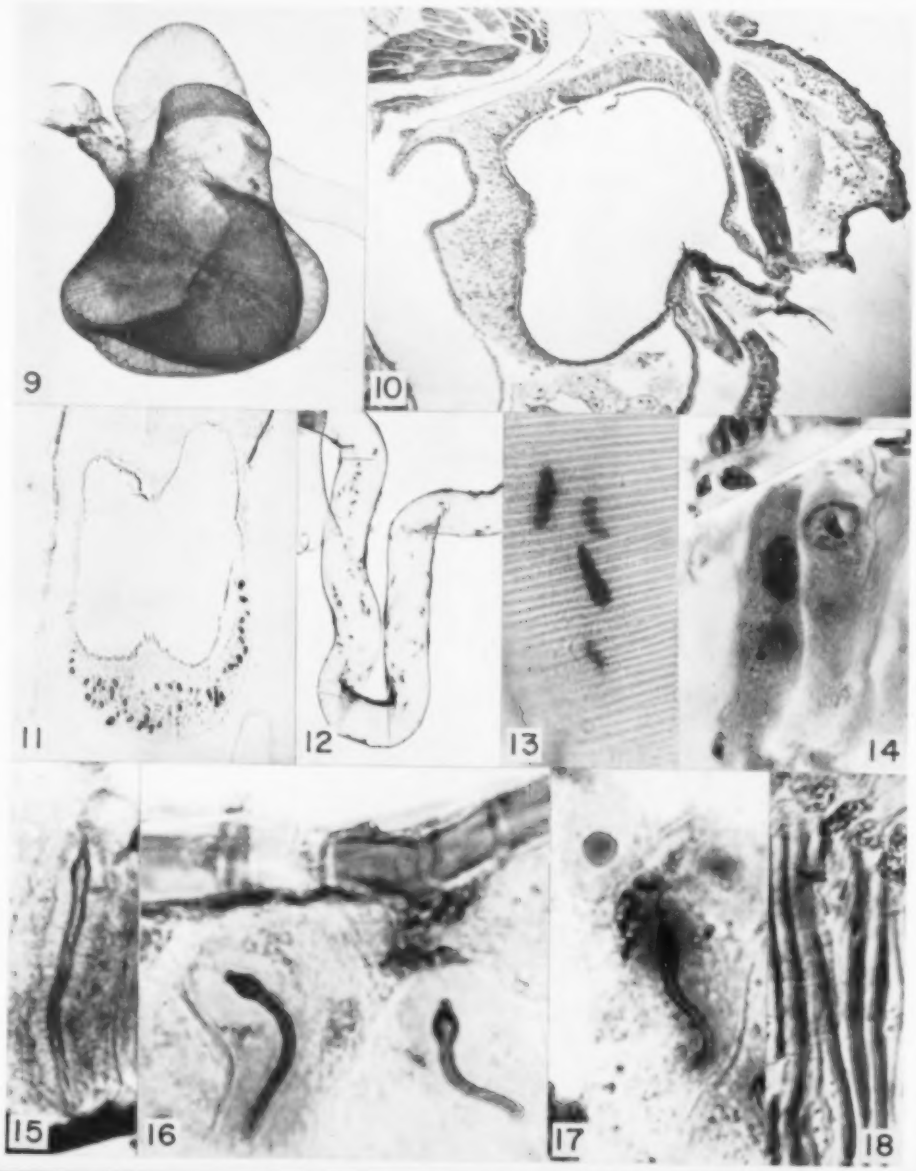


FIGS. 3–5. Tracheal glands of *Diploptera punctata*. (Vertical bar = 1 mm.)

FIG. 3. Dorsal view of the abdomen of a newly emerged adult female from which the wings have been removed. Arrows point to the swellings in the tracheae, which can be seen through the untanned cuticle, near the second abdominal spiracles. FIG. 4. Tracheal glands in situ of a newly emerged adult male. The wings and tergites have been removed. FIG. 5. Right tracheal gland removed from a newly emerged adult male. The long trachea leading from the reservoir to the lateral tracheal trunk is normally coiled (see Fig. 6, CT).



FIGS. 9–18. Histology of tracheal glands near the second abdominal spiracles of adults of *Diploptera punctata*.

FIG. 9. Ventral view of a tracheal gland after treatment with potassium hydroxide. The spiracle is at the left; to the right is the tube leading to the lateral tracheal trunk. × 43. FIG. 10. Median sagittal section of a tracheal gland. A valve separates the spiracular atrium from the neck of the gland. The darkly stained dilator muscle of the spiracle is attached to the valve. At the left is a section of the lateral tracheal trunk at its juncture with the narrower coiled tube (CT of Fig. 6). Both are appressed to the secretory cells on the medio-lateral side of the sac from a newly emerged female. Alcoholic Bouin's fixation, Mallory's triple stain; 10 μ. × 60. FIG. 11. Median longitudinal section of a tracheal gland. The secretory apparatuses are blackened by Gomori's method for alkaline phosphatase. Newly emerged male; 5 μ. × 95. FIG. 12. Portion of the tube leading from the tracheal sac. The secretory apparatuses are blackened. Whole mount treated by Gomori's method for alkaline phosphatase. Two month old male. × 45. FIG. 13. Higher magnification of preparation in Fig. 12. The striated portion of the secretory apparatus reacts for alkaline phosphatase but the bulb does not. × 400. FIG. 14. Columnar secretory cells of the tracheal gland. The nuclei are large with conspicuous nucleoli. Above at right and inset above at left are small nuclei of the cells below the chitinous lining. Note thin convoluted tubule. Three month old female. Orth's fixation; Heidenhain's haematoxylin stain; 5 μ. × 950. FIG. 15. Striated secretory apparatus surrounding a tubule. Note the absence of a large hyaline area around the dilated end of the tubule. Three month old female. Orth's fixation; Fontana's silver stain; 5 μ. × 1500. FIG. 16. Two secretory apparatuses show the hyaline bulb around the dilated part of the inner tubule. The tubules and granules around the bulbs are stained with periodic acid-Schiff's reagent. Above, thin pores penetrate the lining of the sac. Month old adult. Helly's fixation; periodic acid-Schiff's reagent; 5 μ. × 1500. FIG. 17. Blackened granules surround the bulb of the secretory apparatus in a tracheal gland-cell. Three month old female; Orth's fixation; Fontana's silver stain; 5 μ. × 1500. FIG. 18. Portion of a tangential section of the inner wall of a tracheal gland. The dark perpendicular bands are taenidia. Above, two delicate tubules lead to pores between the taenidia. Below, three pores are evident. Newly emerged female. Alcoholic Bouin's fixation; Mallory's triple stain; 10 μ. × 1560.

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tube (Fig. 12). A valve closes the second abdominal spiracle. It is opened by the contraction of a large muscle (Fig. 10) which extends from the valve anteriorly to the lateral sternite and is closed by a small muscle extending between conical processes (Fig. 7, CP) of the rigid part of the valve. No such valve or muscular constriction controls the opening of the sac into the tracheal system. There are no muscles directly associated with the reservoirs.

The tracheal glands of the second abdominal spiracles of nymphs of *D. punctata* also secrete quinones. *Diploptera* differs from the Coleoptera which produce *para*-quinones in large quantities, in that immature stages as well as the adults produce these secretions. Among the beetles which secrete *para*-quinones, only the adults (and very old pupae of *Tribolium*) have been found to produce these materials.

The shape of the cuticular sac varies slightly from the first to the fourth instar of *Diploptera* as shown in the outline drawings of Fig. 19. At each moult the cuticular part of the sac and the quinones within are shed. Occasionally the lining is not shed at the last nymphal moult, resulting in an adult gland with a double lining. No quinone can be demonstrated in the sac of an adult as it emerges from the nymphal skin. Fifteen to thirty minutes later, although yellow fluid is still not visible, the presence of an oxidizing substance is indicated by the formation of a dark red compound when a gland-sac is opened, or squeezed in a solution of dimethyl*para*phenylenediamine hydrochloride. Twenty-four hours after the adult has emerged, globules of yellow fluid are visible in the sac. As the insect ages, the sac becomes progressively more nearly full of quinones, but there is apparently always a small air-filled space in the centre of the sac between the incurrent and excurrent ducts. The sac changes in colour from golden tan in the newly emerged adult to dark brown in adults two months or more old, and the fluid within is sometimes dark yellow rather than light yellow. The coiled tube which connects the sac with the lateral tracheal trunk becomes slightly tan but never dark brown. Perhaps the presence of quinones within the sac causes this darkening and the sac of a newly moulted individual is already tanned by the quinones produced by the gland cells while the rest of the cuticle is still white.

In order to determine whether the glandular cells along the coiled tube also secrete quinones, a loop of the tube from the right gland of twelve newly emerged adults was ligatured with a nylon thread through a small slit made in the second abdominal tergite. Two weeks later the spiracle-gland area was dissected out and examined. In all of the ligatured glands the sacs had partially collapsed. Neither the sac nor the ligatured tube contained yellow fluid; however, the ligatured part of the tubes was brown. Whether this brown coloration of the tubes was caused by an accumulation of quinones or by some injury phenomenon is still an unanswered question. However, it is evident that in order for the sac to maintain its normal expanded shape it must be connected with the main tracheal system.

The cuticular part of the sac, which remains after treatment with potassium hydroxide, is shown in Fig. 9. Characteristic taenidia and, in some areas, hair-like processes are found. Histological sections of the tracheal sac of the adult

reveal that the glandular tissue is composed of many layers of secretory cells (Fig. 10). The taenidial "hairs" occur in areas where the epithelium is only one cell-layer thick (Fig. 10). The boundaries of the secretory cells are not easily distinguishable. The cells shown in Fig. 14 indicate that the secretory cells are columnar and the nuclei are large with conspicuous nucleoli. Cells with small nuclei underlie the chitinous lining (Fig. 14) and are intermingled with the large

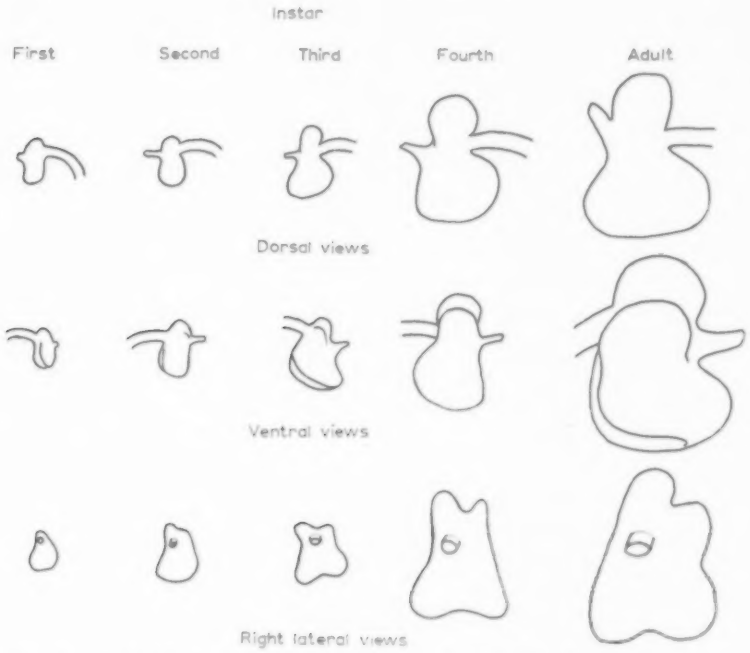

FIG. 19. Camera lucida outline drawings of the tracheal glands of the various instars and adult of *Diploptera*.

secretory cells. The secretory apparatus characteristic of these glandular cells is composed of a striated cylinder and a hyaline bulb surrounding a narrow tube which is slightly dilated within the bulb and then becomes a very thin tubule (Fig. 16). Sometimes the hyaline bulb surrounding the dilated tube is absent (Fig. 15). The striated cylinder is often coiled and therefore a section through its entire length is rare. The very thin tubules convey the secretion through small pores between the taenidia. Several pores occur in one area where the taenidia diverge slightly from their parallel orientation (Fig. 18).

The cytology of the secretory cells of the odoriferous glands of *Diploptera* is much like that of *Tribolium*. PALM (1946) described two types of secretory cells in *Tribolium destructor*. The one-cell units adjacent to the storage bladder contain winding canals surrounded by hyaline areas of cytoplasm. The two-cell units

composing the branched lobes of the gland possess a canal which passes through a rhabdorium or striated vesicle in one cell, and then through a hyaline area in the second cell, and on through other cells to the reservoir. Within the rhabdorium the canal is flask-shaped; within the hyaline area it has diverticula. Similarly in *Tribolium confusum*, ROTH (1943) described single cells with canals passing through hyaline areas of cytoplasm and aggregates of cells with canals passing through two vesicles, one striated and the other hyaline. ROTH did not find these two vesicles occurring within two separate cells. In *Diploptera* the two vesicles of the secretory apparatus seem to be within one cell, and the shape of the canal or tube within the apparatus is more like that described for *T. confusum* than for *T. destructor*.

The histology of the glandular cells of the nymphal tracheal swellings of *Diploptera* is essentially the same as that of the adult gland.

When observed under ultra-violet light the glandular tissue around the sac fluoresces strongly yellow. Sections of the gland stained with Sudan black B after fixation in 10 per cent formalin reveal only small particles of fat around the secretory apparatuses. Glycogen, i.e. that material which stained with periodic acid-Schiff's reagent (PAS) and was removed by treatment with saliva, occurs in abundance in the gland cells of newly emerged individuals, but not in those several days old or older. However, glycogen was found in the cells of the glands mentioned earlier, which were ligatured from the main tracheal system. The tubule within the entire secretory apparatus is stained red with PAS (Fig. 16). Granules around the secretory apparatus are also stained with PAS especially well after a dichromate fixative (Fig. 16). The granules are not well preserved with alcoholic Bouin's fixative or with 95 per cent ethyl alcohol and are more prominent in glands from old individuals than newly emerged ones. Fontana's silver stain after dichromate fixation also reveals granules around the secretory apparatus (Fig. 17). The striated part of the secretory apparatus gives a strongly positive reaction with Gomori's test for alkaline phosphatase (Figs. 11–13). This is a characteristic reaction of such secretory structures as those of the colleterial glands of *Periplaneta americana* (BRUNET, 1952; KUGLER *et al.*, 1956) and the scent gland of *Eurycotis floridana* (STAY, 1957).

The glandular cells do not react as strongly with Nadi reagent as does muscle tissue. However, small blue granules do form in the cytoplasm of the secretory cells and such colour formation is inhibited by 0·002 M potassium cyanide or sodium azide. Blue granules were not found in the striated or hyaline part of the secretory apparatus, but were sometimes observed within the central canal of the secretory apparatus and in the small tubule leading to the lumen. Quinones readily oxidize the Nadi reagent to indophenol blue, even in the presence of azide and cyanide. Perhaps these granules indicate the presence of quinones on the way to the storage sac.

The reaction of the granules surrounding the secretory apparatus with ammoniacal silver solution and periodic acid-Schiff's reagent after dichromate fixation suggests that these secretory granules are phenolic compounds which may be precursors of the quinones in the sacs.

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BRUNET and KENT (1955) have shown that the *ortho*-quinone produced by the colleterial glands of *Periplaneta americana* is formed from the β-glucoside of its phenolic precursor. A β-glucosidase hydrolyses the β-glucoside and a phenol oxidase then oxidizes the phenol to the quinone-tanning agent.

The presence of a β-glucosidase in the tracheal glands was demonstrated by incubating fresh whole glands from newly emerged *Diploptera* with salicin, in acetate buffer pH 4·8 for 16 hr at 37° C. A drop of 1 per cent merthiolate was added to prevent contamination. The incubated solutions were analysed by descending paper chromatography. Whatman No. 1 filter paper was spotted with the test solutions and with known substances and run at room temperature in *iso*-propanol : acetic acid : water (54 : 8 : 18). The papers were dried and sprayed with either ferric chloride : potassium ferricyanide : water (1 : 1 : 100) which brings out phenols, or sprayed with benzidene : acetic acid : ethanol (1 : 4 : 16) which brings out reducing sugars. Glucose and saligenin, the hydrolysis products of salicin, were demonstrated when salicin was incubated with tracheal glands. Tracheal glands alone in buffer showed no saligenin and only a small amount of glucose. No change occurred in salicin incubated alone. Heating the glands in buffer at 90° C for 10 min did not inactivate the enzyme, but boiling the glands in water for 5 min did destroy their ability to hydrolyse salicin. Treating the glands with 95 per cent ethanol before incubating them with salicin, also inactivated the enzyme.

The dinitrosalicylic acid (DNS) method of SUMNER and SOMERS (1944) for reducing sugars was used to measure, quantitatively, the glucose produced in the hydrolysis of salicin. Twenty glands, from newly emerged adults, incubated in 0·5 cm^3 of a 0·5 per cent solution of salicin (in 0·05 M acetate buffer pH 4·8 for 16 hr at 37° C) gave 15 per cent hydrolysis of the salicin.

The β-glucoside extracted from the left colleterial glands of *Eurycotis floridana* was hydrolysed by whole fresh spiracle glands of *Diploptera*. The products, identified by paper chromatograms, were glucose and protocatechuic acid. Thus the tracheal glands possess a β-glucosidase capable of hydrolysing salicin as well as the β-glucoside from the colleterial glands of *Eurycotis floridana*.

The presence of a polyphenol oxidase in the gland cells was demonstrated by the oxidation of catechol following the method of SMYTH (1954). Spiracle glands were removed from newly emerged adults and fixed overnight in 70 per cent ethanol. The tissues were rinsed in water and incubated for 45 min at 40° C in a 0·2 per cent solution of catecol. The gland cells became dark pink. The colour which was not localized in any particular part of the cell was destroyed by treatment with 0·1 per cent sodium hypochlorite. Sodium azide (0·002 M), potassium cyanide (0·002 M), and phenylthiourea (saturated solution) inhibited the coloration of the secretory cells. Thus, the secretory cells of the spiracle gland also exhibit polyphenol oxidase activity. Whereas a β-glucosidase and a polyphenol oxidase have been demonstrated, as yet a β-glucoside has not been identified from ethanolic or water extracts of the tracheal glands. One can only surmise that the *para*-quinones of the tracheal glands of *Diploptera punctata* are formed in the same way as the *ortho*-quinones of the colleterial glands of *Periplaneta americana*.

Other tracheal glands in cockroaches. It will be noted that the trachea leading to the third abdominal spiracle of *Diploptera* is bent upon itself between the lateral tracheal trunk and the spiracular opening (Fig. 6, T3). Likewise, the trachea of the seventh abdominal spiracle is bent upon itself and enlarged near the spiracle. Secretory cells are associated with the modifications of the tracheae near the third and seventh abdominal spiracles, but the tracheal lining is not tanned as is the sac of the second segment, nor is any secretion visible. The glandular cells of these tracheal modifications appear to have a simpler secretory apparatus than those of the quinone-producing gland.

In the Blattaria, enlargement of the tracheae adjacent to spiracles is not peculiar to *Diploptera*. Swellings with closely related shapes to those of *Diploptera* occur in the corresponding segments of *Blaberus craniifer* Burmeister, *Blaberus giganteus* (Linnaeus), and *Byrsotria fumigata* (Guérin). No coloured liquid has been found in these swellings, in the few specimens examined, although a dark red solid sometimes is present in the tracheal enlargements of *Byrsotria*. In *Pycnoscelus surinamensis* (Linnaeus), modifications of the tracheae are evident only near the second abdominal spiracles. *Panchlora nivea* (Linnaeus) has a small enlargement in the trachea near the second abdominal spiracle and larger swellings near the sixth and seventh abdominal spiracles.

In *Leucophaea maderae* (Fabricius) and in *Nauphoeta cinerea* (Olivier) the tracheae leading from the eighth abdominal spiracles are coiled several times before they enter the lateral tracheal trunks.

Wolcott (1924) has pointed out that *Leucophaea maderae* ordinarily does not impart a bad odour to the substances with which it comes in contact, nor do its excreta have a bad odour; however, on being disturbed this cockroach readily emits a strong and disagreeable odour. The tracheae near the second abdominal spiracles of *L. maderae* are not modified in shape but the cells in this region appear much whiter than ordinary tracheal epithelium. These whitish gland cells produce the odorous material which is so characteristic of *Leucophaea maderae*. Nymphs (including early instars) as well as adults emit this odour when they are disturbed. Thus in *Leucophaea* as well as *Diploptera* odorous substances are emitted from the second abdominal spiracles. The nature of the substance secreted by *L. maderae* is unknown, although apparently it is not quinone-like.

All of the previously mentioned cockroaches which possess modified tracheae are the so-called ovoviviparous species which incubate their eggs internally. Eight oviparous species were examined and only one exhibited tracheal swellings near the spiracles. *Eurycotis floridana* (Walker) has two swellings in the trachea, near the eighth abdominal spiracle, which are light tan in colour and are surrounded by secretory cells. No modifications in the vicinity of the abdominal spiracles were found in *Periplaneta brunnea* Burmeister, *Blatta orientalis* Linnaeus, *Blattella germanica* (Linnaeus), *Blattella vaga* Hebard, *Neostylopyga rhombifolia* (Stoll), *Parcoblatta virginica* (Brunner), and *Supella supellectilium* (Serville).

It may be mentioned here that the grasshopper *Romalea microptera* (Beauv.) (= *Dictyophorus reticulatus* Thunberg), found in the south-eastern United States,

when disturbed, gives off with a hissing sound, a disagreeable dark brown, frothy liquid, from the mesothoracic spiracles (MORSE, 1907).

DISCUSSION

Possible function of the quinones emitted by arthropods. The function of the *para*-quinones in *Diploptera* and the other arthropods in which they occur is unknown. The quinones of the tracheal sacs of *Diploptera* are not concerned with the general tanning process of the cuticle since both sacs were removed from six nymphs in the last instar and these insects continued to adult development and normal tanning in the absence of their tracheal glands.

The emission of quinones by arthropods when they are disturbed or attacked can be considered a defensive weapon against predators. Yet, in Hawaii, *Diploptera* is reported to be eaten by the cockroach *Nauphoeta cinerea* (ILLINGWORTH, 1942), the toad *Bufo marinus* (PEMBERTON and WILLIAMS, 1938), and possibly by mynah birds (ILLINGWORTH, 1928). In the laboratory, we have fed *Diploptera* to praying mantids and toads which will readily accept them as food. However, the fact that certain animals will feed on *Diploptera* does not preclude the possibility that the quinone-secreting tracheal glands are protective in function. Our knowledge of the relationships of *D. punctata* to other animals in the field is meagre, and a study of its ecology might reveal the presence of certain predators, in the cockroach's environment, which are repelled by quinones. Dr. THOMAS EISNER (personal communication) of Harvard University is currently studying the behaviour of ants towards *Diploptera*, in the laboratory. He has found that *Pogonomyrmex badius* (Latreille) avoided *Diploptera* nymphs and adults as soon as they ejected the slightest trace of quinones. *Diploptera* from which the tracheal glands had been removed were attacked by the ants and a moulting or freshly moulted *Diploptera* was also vulnerable.

One might consider the quinones as playing a more subtle physiological role, particularly in *Diploptera*, where these biologically active compounds are secreted into a structure which is part of the respiratory system. However, Dr. JAMES F. CASE (personal communication) has investigated the innervation of the muscles controlling the opening and closing of the second abdominal spiracle of *Diploptera* and he has commented on this gland as follows: ". . . while the gland is open to the general tracheal system, its contents probably do not enter the respiratory tract because the spiracular valve remains closed during the entire respiratory cycle. When the valve does open my impression is that it does so only upon expiration, which, if forceful enough, causes ejection of quinone-containing fluid as air is forced from the longitudinal tracheal trunk through the secretory ampulla. Thus the rather long tracheal connection between the secretory ampulla and longitudinal tracheal trunk seems to be a reasonable solution to the problem of keeping the quinone out of the tracheal system, while at the same time permitting utilization of the force of expiration to eject the contents of the gland. I have wondered whether or not ejection is also facilitated by simultaneous closure of all other spiracles but have not been able to decide either way."

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ACETYLCHOLINE SYNTHESIS IN THE HOUSEFLY HEAD

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Abstract—The acetylcholine-like substance which is synthesized by fly head acetone powder extracts, with and without the addition of choline and acetate as substrates, was shown to behave chemically and chromatographically as acetylcholine. The biological and chemical quantitative estimation of the synthesized substance agreed. Analogues of acetate were tried as substrates in addition to choline, and the product of the synthesis was analysed chromatographically. The corresponding ester was formed only with propionate; with formate and butyrate only, the spot of acetylcholine appeared. The mechanism of the synthesis of acetylcholine without any addition of substrates was compared with that occurring with added choline and acetate. No substantial difference was found as regards the pH/activity curve, the time/activity curve, and the requirements for cysteine, CoA, and ATP. Phosphorylcholine was tried instead of choline, but was found ineffective as substrate for the synthesis. The significance of acetylcholine in the nervous transmission of insects is discussed.

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Both from a physiological and from a practical point of view it is important to determine if acetylcholine is the active substance in the nervous transmission of insects, and if its synthesis follows the same pattern as is known to occur in the vertebrate nervous system. The identity of the acetylcholine-like substance present in the insect nervous system, still questioned by many authors, was analysed by paper chromatography. Thus Chefurka and Smallman (1955) found acetylcholine in extracts of housefly heads, and Augustinsson and Grahn (1954) found in extracts of bee heads at least three principles active on the guinea-pig ileum: acetylcholine, histamine, and a third unidentified substance. It may be mentioned that not only in insect but also in vertebrate tissues, paper chromatography has revealed the presence of substances with acetylcholine-like activity, other than acetylcholine itself (Banister, Whittaker, and Wijesundera, 1953; Schümann, 1955).

The mechanism of acetylcholine synthesis was studied by Smallman (1956) in the blowfly by adding choline and either acetate or citrate as substrates, and it was found to proceed by a two step reaction (acetyl-CoA formation and acetylation of choline) as in the vertebrate's brain. Without any addition of substrates there is also a synthesis of an acetylcholine-like substance (Lewis, 1953), but its identity with acetylcholine has not yet been fully demonstrated. If it is acetylcholine, the mechanism of its synthesis could also follow some alternative pattern, different from that already demonstrated. These two questions are the subject of the present investigations.

MATERIALS AND METHODS

Houseflies were reared to pupation on a culture medium composed of wheat bran, water, and milk (3 : 4 : 2), and the adults were provided with water and sucrose until they were killed 5–10 days after emergence. They were anaesthetized with ether, decapitated on dry

ice in lots of 2–3 thousand, and acetone dried powders were prepared from the heads at a temperature of −5°C to −3°C.

The powders were extracted with a solution having the following composition: KCl 0·17 M, $MgCl_2$ 0·0025 M, Na_2HPO_4 0·006 M (2·5 ml every 100 mg of powder) during 60 min at 10°C, and the suspension centrifuged for 10 min at 14,000 rev/min. The reaction mixture contained μM per ml (final concentration): cysteine 10, ATP 3, $MgCl_2$ 1·5, KCl 85, Na_2HPO_4 3, TEPP 0·35; CoA 2–4 units per ml. Choline chloride and sodium acetate or propionate or butyrate when present had a final concentration of 10 μM per ml. Incubation was carried out in small test tubes in a thermostat at 37°C, and the reaction stopped by deproteinization with 0·1 ml N HCl per ml (neutralized after 20 min with 0·1 ml N NaOH).

The determination of acetylcholine was carried out biologically on the guinea-pig ileum, and, in some cases, chemically by the method of HESTRIN (1949).

For chromatography, eserine (1 μM per ml) was used instead of TEPP, because the latter substance caused the appearance of two spots for acetylcholine (the first with the usual R_f value of 0·47, the second with a value of 0·65). After deproteinization the reaction mixture was lyophilized and extracted with ethanol. The ethanol was concentrated by means of a warm air stream and distributed on a strip of Whatman No. 4 filter paper. In other cases deproteinization was carried out directly with ethanol, and the supernatant concentrated in the same way. The solvent used was *n*-butanol/ethanol/acetic acid/water (8/2/1/3) (AUGUSTINSSON and GRAHN, 1953), with the descending technique. The spots were revealed on the paper by spraying with a solution of dipicrylamine (MALYOTH and STEIN, 1951), with a modification of Dragendorff's reagent (NOVELLI and GUERRERO, 1953), with the hydroxylamine-ferric chloride method (WHITTAKER and WIJESUNDERA, 1952), or biologically by direct immersion of paper strip sections in the guinea-pig ileum bath.

Paper electrophoresis was carried out in a horizontal apparatus 18 cm long and 4 cm wide. Whatman No. 1 filter paper strips were used, with 5 per cent acetic acid at 300 V during 30 min.

Preparation of aged extracts from pigeon liver acetone powder and acetylation of sulphanilamide were performed according to NOVELLI (1955).

CoA (70 per cent pure) was obtained from the Bios Laboratories, and phosphorylcholine chloride (barium salt) from the California Foundation for Biochemical Research.

RESULTS

(1) *Identity of the acetylcholine-like substance synthesized by fly head acetone powder extracts*

Fig. 1 summarizes the chromatographic experiments performed, and shows that the product of the synthesis with and without substrates has exactly the same

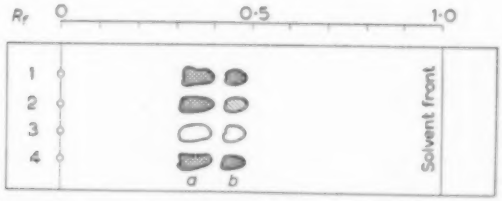

FIG. 1. Chromatography preceded by electrophoresis of a fly head acetone powder extract after incubation: (1) with choline and acetate, (2) with choline, (3) without substrates. (4) inactivated extract + choline (a) and acetylcholine (b). Development with Dragendorff's reagent. The weight of the shading corresponds to the intensity of the colour.

R_f as acetylcholine. A positive reaction is obtained with dipicrylamine, Dragendorff's reagent, the hydroxylamine-ferric chloride method, and the guinea-pig ileum.

When the synthesis is small, as is the case in the absence of substrates, a high concentration of the extract has to be put on the paper in order to reveal the spots; and this was the reason for earlier contradictory results (FRONTALI, 1956). The high concentration of interfering substances on the paper slowed the run of choline and acetylcholine and gave smaller R_f values for both of these compounds. When chromatography was preceded by electrophoresis (the dipicrylamine positive band was eluted with methanol, concentrated, and put on paper), the interfering substances were removed, and the preformed fly head choline and the acetylcholine synthesized in the absence of substrates gave R_f values identical with those of the pure substances.

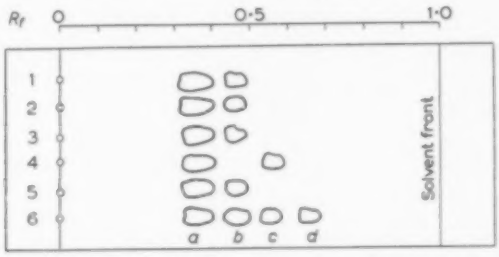

FIG. 2. Chromatography of fly head acetone powder extract after incubation with: (1) choline, (2) choline and formate, (3) choline and acetate, (4) choline and propionate, (5) choline and butyrate, (6) inactivated extract + choline (a), acetylcholine (b), propionylcholine (c), and butyrylcholine (d). Development with Dragendorff's reagent.

The results of synthesis with analogues of acetate as substrates were also examined chromatographically. They are summarized in Fig. 2. With propionate as substrate only one spot appeared, having the same R_f as pure propionylcholine; traces of acetylcholine (R_f 0·47) were revealed by the much more sensitive biological test. With formate and with butyrate as substrates only, the spot of acetylcholine appeared. In this case chromatography was not preceded by electrophoresis, but deproteinization with ethanol was adopted, as this method also eliminates much of the interfering material.

An extract from fresh fly heads was deproteinized and run chromatographically. The strip of paper was cut in sections, and every section was assayed as substrate in addition to acetate for acetylcholine-like substance synthesis. Only the section corresponding to the R_f of choline resulted in an increased synthesis, thus showing that choline is probably the naturally occurring substrate for the synthesis, and that the naturally synthesized product is acetylcholine.

Another method of assaying the identity of the synthesized substance was to compare the results of the biological with the chemical method of Hestrin. Table 1 shows a good correspondence between the two methods, whereas a discrepancy would have indicated the presence of a substance other than acetylcholine.

TABLE 1—ACETYLCHOLINE SYNTHESIS (mg/hr g ACETONE POWDER) WITH AND WITHOUT SUBSTRATES

Method	Substrates			
	Choline and acetate	Choline	Acetate	–
Guinea-pig ileum	3·0	1·5	0·6	0·4
Hestrin	2·7	1·4	0·8	0·3

(2) *Mechanism of the acetylcholine synthesis by fly head acetone powder extract without addition of substrates*

Acetylcholine synthesis has been determined in relation to the pH of the medium (Fig. 3). Both with and without substrates the resultant curves were very

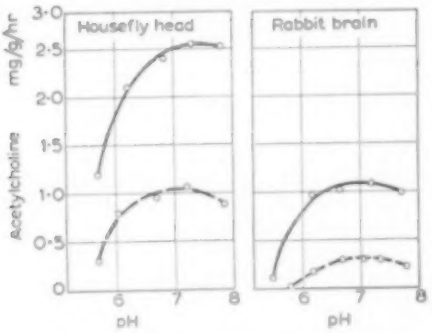

FIG. 3. Acetylcholine synthesis by acetone powder extracts ——— with substrates, — — — — without substrates.

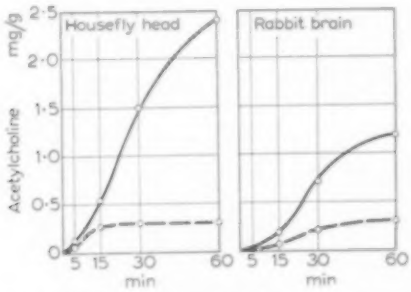

FIG. 4. Acetylcholine synthesis by acetone powder extracts ——— with substrates, — — — — without substrates.

similar to that obtained by SMALLMAN (1956) with acetate as acetyl donor, but were different from that obtained with citrate as acetyl donor by the same author (optimum pH around 6·3).

The time/activity curves with and without substrates for fly head and rabbit brain acetone powder extracts are shown in Fig. 4. KOREY, DE BRAGANZA, and NACHMANSOHN (1951), using squid ganglia acetone powder extracts with choline and acetate as substrates, obtained an S-shaped time/activity curve indicating a reaction which is required for and precedes the acetylation of choline; this reaction was later identified with the acetylation of CoA. In the present experiments S-shaped time/activity curves were obtained both with and without added substrates, but the first period, strongly limited by the activity rate of the acetyl donor system, was shorter with the housefly (5 min) than with the rabbit preparation (15 min). KOREY and his collaborators also obtained a first period of 15 min with the squid preparation.

From Figs. 3 and 4 it may be observed that the fly preparation is much more active than the rabbit's, although it is carried out with whole heads and not with nervous tissue alone. Its activity corresponds to that obtained by SMALLMAN (1956) for blowflies at the same CoA concentration (2·5 mg acetylcholine/g hr with 2 units of CoA per ml).

The activity of the acetyl donor system was assayed in the fly head acetone powder extract also by means of the acetylation of sulphanilamide. Table 2 shows

TABLE 2—ACETYLATION OF SULPHANILAMIDE BY DIFFERENT PREPARATIONS

Extract from 25 mg acetone powder	CoA u/ml	Sulphanilamide μg acetylated in 90 min
Fly head	2	5·6
Fly head	—	4·2
Pigeon liver	2	69·0

that fly head acetone powder extracts are about 12 times less active than pigeon liver's in acetylating sulphanilamide. Without any addition of CoA, acetylation is 75 per cent of the optimal, owing to CoA already present.

The enzyme system synthesizing acetylcholine from choline and acetate requires, as the corresponding rabbit brain system, ATP and CoA. Without the addition of substrates, the synthesis is not influenced by the presence of ATP and

TABLE 3—ACETYLCHOLINE SYNTHESIS (mg/hr g ACETONE POWDER) WITH AND WITHOUT ATP AND CoA (4 u/ml). CYSTEINE ALWAYS PRESENT

With substrates +	CoA + ATP	6·0
	CoA	0·7
	ATP	0·7
	—	0·6
Without substrates +	CoA + ATP	0·3
	CoA	0·3
	ATP	0·3
	—	0·3

CoA (Table 3), clearly because these compounds are already present in an amount which is sufficient for such a low synthesis as may take place with the choline and acetate already present in the extract. These experiments were carried out with cysteine; in the absence of cysteine, CoA, ATP, and substrates the acetylcholine synthesis was about 0·1 mg/g hr.

Phosphorylcholine has been used instead of choline as substrate for acetylcholine synthesis, but it did not appear to be utilized. Phosphorylcholine was found to enhance the sensitivity of the guinea-pig ileum to acetylcholine.

DISCUSSION

The doubts concerning the mechanism of nervous conduction in insects, and the identity of the acetylcholine-like substance present in the insect nervous system, arose mainly as a result of the discovery that acetylcholine and its antagonists atropine, scopolamine, and curare were almost ineffective on roach ganglia (ROEDER, 1948). Also the injection of as much as 1 mg/g of acetylcholine and of other choline esters in roaches and grasshoppers produced no significant physiological response (TOBIAS, KOLLROS and SAVIT, 1946; HOPF, 1952). ROEDER (1948) suggested two possible explanations: (1) the inability of these substances to penetrate the synapse and (2) the occurrence of a different chemical transmitter in the insect nervous system. This second hypothesis appeared the more likely according to ROEDER. Some experiments performed by FERNANDO (KEARNS, 1952) seemed to confirm this second hypothesis: the biologically active substance present in a roach nervous tissue extract did not show a response to HESTRIN's chemical test for acetylcholine, and it caused the isolated roach leg to contract, whereas acetylcholine when administered at much higher concentrations had no such effect. On the other hand, the first hypothesis was recently confirmed by ROEDER himself (TWAROG and ROEDER, 1956). Thus desheathing the roach abdominal ganglia enabled synaptic transmission to be influenced by acetylcholine and other pharmacological substances.

Much work has been done on substrate preferences of insect cholinesterases, mainly in connexion with the studies on the mode of action of anticholinesterase drugs, and acetylcholine has often been questioned as the natural substrate (CHADWICK and HILL, 1947; HOPF, 1954). Recent work (METCALF, MARCH and MAXON, 1955), however, shows that all the insect heads investigated contain, in addition to other esterases, a specific acetylcholine-esterase with higher activity for acetylcholine than for any other choline ester. Thus the hypothesis of acetylcholine as the active substance in insect nervous transmission seems the most probable.

The present work supports this hypothesis, as the acetylcholine-like substance which is synthesized from already present substrates is shown to behave chemically and chromatographically as acetylcholine. LEWIS (1953) chromatographed the active substance synthesized by blowfly head extracts without any addition, and found no detectable separation of it from the added acetylcholine. This active substance, however, is not said to have an R_f corresponding to that of pure acetylcholine, and in fact we found that under certain conditions this correspondence is disturbed by

interfering substances. This inconvenience is overcome by the ethanol deproteinization method or by chromatography preceded by electrophoresis. The active substance was found to give the Hestrin reaction and the biological and chemical assay agreed.

Analogues of acetate (already bound to CoA) have been tried by BERMAN, WILSON and NACHMANSOHN (1953) as substrates for purified squid ganglia choline acetylase; propionyl-CoA was found to react approximately at the same rate as acetyl-CoA, and butyryl-CoA showed almost no reactivity, although it was bound to the enzyme even more strongly than acetyl-CoA. The same was true for purified acetylcholine-esterase. Propionylcholine was hydrolysed almost at the same rate as acetylcholine, whereas butyrylcholine was bound to the enzyme but was hydrolysed at a very slow rate. Our experiments are in agreement with BERMAN *et al.*, as they show that only propionate gives rise to the corresponding ester. As regards cholinesterase it may be noted that housefly head homogenates hydrolyse butyrylcholine at a still higher rate than acetylcholine (METCALF, MARCH and MAXON, 1955), but this hydrolysis follows an S-shaped activity/substrate concentration curve and is probably due to an unspecific esterase present in addition to acetylcholinesterase in the housefly head.

As regards the mechanism of acetylcholine synthesis from the substrates already present in the extract, the pH/activity curve has been shown to be parallel to that occurring when acetate is added as acetyl donor and very different from that occurring when citrate is added (SMALLMAN, 1956). This would suggest that the natural acetyl donor system is in this case prevailingly that catalysed by acetyl-CoA-kinase, and not by the condensing enzyme. The acetyl donor system in the fly head acetone powder extract seems to be more active than the rabbit brain preparation, as can be seen from the shorter induction period in the time/activity curves. The reaction without addition of substrates requires cysteine; if no additions of CoA and ATP are needed, this is clearly due to sufficient amounts of these substances already present in the extract.

BERRY and STOTZ (1953) and KOMETIANI (1952) found that phosphorylcholine could be used as a substrate for acetylcholine synthesis in brain acetone powder extracts or homogenates instead of choline, but with our preparation we could not confirm these results.

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CYCLIC CO_2 RELEASE IN DIAPAUSING PUPAE—II

TRACHEAL ANATOMY, VOLUME AND pCO_2; BLOOD VOLUME; INTERBURST CO_2 RELEASE RATE

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Abstract—An average one gram cyclically-respiring diapausing pupa of the saturniid moth *Agapema galbina* has a tracheal volume of about 60 μl and a tracheal pCO_2 of 45 mm Hg. This shows that nearly 90 per cent of the approximately 30 μl of gaseous CO_2 released during a "burst" must come out of solution and combination in tissue stores. Body water per g is distributed: blood, 330 μl; tissues, 180 μl; gut lumen, 100 μl; and cuticle 110 μl. Dimensions of the major parts of the tracheal path from environment to tissues are given, and it is shown by computation that the spiracular valve is the only site where significant resistance to diffusion could occur. It is shown statistically that the rate of CO_2 release increases gradually during the interburst period, as it should if CO_2 is being impounded in body buffers. Arguments are advanced for cyclic CO_2 release being potentially widespread in insects under conditions where O_2 supply is high relative to demand.

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INTRODUCTION

PREVIOUS work on the remarkable "bursts" of CO_2 released periodically by certain insects has yielded a considerable body of facts (for review see SCHNEIDERMAN and WILLIAMS, 1955; BUCK and KEISTER, 1955), but several data essential for a quantitative description and understanding of the physical mechanism of CO_2 retention are still lacking. Among these are the volume of the tracheal system, the dimensions of the environment-tissue diffusion pathway, pupal blood and tissue water content, and the composition of the tracheal gas during the cycle. The present paper deals with attempts to supply these and certain other informational deficiencies. Most of our data are derived from diapausing pupae of the saturniid moth *Agapema galbina*, but we wish particularly to thank Dr. HOWARD SCHNEIDERMAN for sending us Cecropia pupae (*Hyalophora*) for comparison.

1. TRACHEAL VOLUME

As an indication of the volume of endogenous gaseous CO_2 which is immediately available for release, in contrast to that which has first to diffuse out of tissue liquids, the volume of the pupal tracheal system is an important datum. SCHNEIDERMAN and WILLIAMS have reported (of *Hyalophora*) that "Vacuum extraction of the gases in the tracheal system and dissolved in the tissue fluids

revealed the tracheal volume of Cecropia pupae to be considerably less than 10 per cent of the total volume of the insect". In our opinion this, the usual method of estimating tracheal volume, is subject to two potentially serious sources of error. The first is that one has no way of knowing how much of the extracted gas is normally present as gas (i.e. in the tracheae) and how much is evacuated from solution and combination in blood and tissues. The second objection is that evacuation under water (as is usually done) does not remove by any means all of the gas in the pupal tracheal system. This is seen very clearly in the fact that the abdomen elongates greatly when the vacuum is applied, due to expansion of trapped tracheal gas, and then shortens when atmospheric pressure again prevails, and that this reversible telescoping continues with repeated applications of vacuum

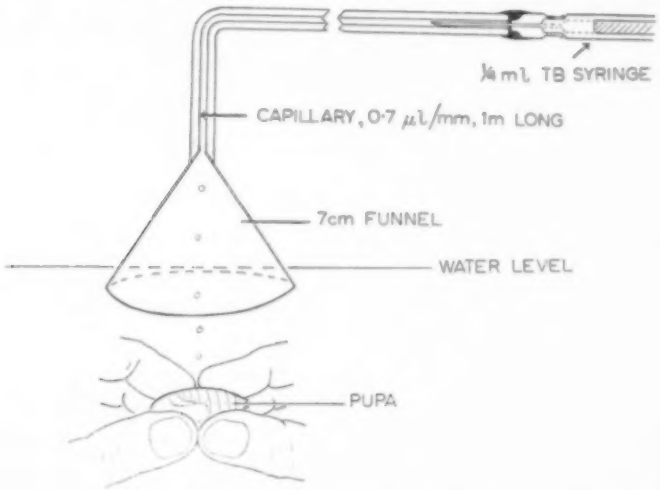

FIG. 1. Apparatus for measuring volume of expelled tracheal gas (see text for details).

regardless of whether or not gas bubbles actually issue from the spiracles during the first evacuation.

Our method for measuring tracheal volume is based on the fact that in many insects a relatively small mechanical pressure applied to the exoskeleton and transmitted through the body liquids will completely collapse the tracheal system, driving the tracheal gas out through the spiracles where its volume can be measured. In practice this was accomplished as follows: The pupa was first picked up between the thumb and first two fingers of both hands; the fingers and pupa were then quickly dipped in 70 per cent alcohol (to prevent adhesion of small air bubbles), then immediately immersed in water directly under the liquid-filled capillary-funnel arrangement shown in Fig. 1. Often within a few seconds the pupa contracted its abdomen spontaneously, driving gas bubbles out of the spiracles. After this occurred, or if it did not occur, the pupa was compressed firmly in a direction parallel to its longitudinal axis to drive out any remaining gas. The gas bubbles

issuing from the spiracles were brushed off the pupa with the fingers if they did not detach spontaneously, and the total volume of gas accumulated at the funnel-capillary junction was then drawn into the horizontal part of the calibrated capillary by means of the syringe cemented to the end of the capillary. Finally the length of capillary liquid displaced was measured. The water with which the immersion basin, funnel and capillary were filled contained NaCl to saturation at 25°C (to reduce gas solubility), dilute HCl (to reduce solution of CO_2), and a few drops of caprylic alcohol (to prevent foaming and to reduce possible resistance to gas escape by the capillarity of the air–water interface at the spiracles).

It was found that after the tracheal system was emptied of gas expellable by "reasonable" pressure, an additional small amount could sometimes be obtained by pressure extreme enough to explode and completely eviscerate the animal. This procedure is not considered desirable, both because of the trapping of gas froth in the viscera floating up into the funnel, and because of the possibility that if gas were present elsewhere in the body than the tracheae—for example, in the gut or under the wings—it might be added to the tracheal gas. Another precaution taken was to avoid pupae with parasites, since in these very large extratracheal, gas spaces may occur if the parasite has started development (Buck and Keister, 1956).

If there were significant physical resistance to gas escape at the spiracles our method of measuring tracheal volume would be vulnerable to the objection that the pressure might have driven some of the tracheal gas into solution in the body liquids rather than to the exterior. As a check against this possibility we also squeezed pupae which had been in flowing pure N_2 for ½–2 hr just previously, a treatment that should have had the dual effects of forcing the spiracular valves to open and of replacing all the tracheal and tissue gases with a relatively insoluble single gas.

Table 1 summarizes the results obtained with *Agapema* pupae. It will be seen that there was no consistent difference between pupae pre-treated with N_2 and those not treated, but that there was a great variation between individuals in volume of expressible gas. This variability is due, we believe, almost entirely to variation in degree of abdominal extension at the moment of submersion, low values presumably representing pupae which had contracted before being measured. This is suggested also by the complete lack of correlation between body weight and tracheal gas volume. Values marked with a dagger (†) represent pupae that were squeezed to complete evisceration. These indicate that no characteristically greater gas volume is obtained by this extreme procedure. The values marked with asterisks are regarded as particularly reliable in the sense that the gas was expelled with ease.

In summary, it can be concluded that the tracheal volume of normal *Agapema* pupae varies, and probably is capable of changing in the individual, at least over the range 10–75 μl/g, and that a reasonable average figure for non-contracted individuals might be of the order of 62 μl/g (mean for the 7 "best" determinations). This would correspond to about 6 per cent of body volume, assuming an average tissue specific gravity of 1·0. Determinations on two Cecropia pupae gave a figure of 7·5 per cent. Determinations on three *Bombyx* pupae (one 16, one 9, and one 8

days after spinning) gave the low value of 0·8 per cent. Since in *Agapema* about 30 μl of CO_2 are liberated in an average burst, the concentration of CO_2 in the tracheal gas would need to be almost 50 per cent if the burst represented tracheal

TABLE 1—TRACHEAL VOLUME IN 32 DIAPAUSING *Agapema* PUPAE

	Individual tracheal volumes above (μl/g) and weights below (mg)							
No N_2 pre-treatment	67*	19	56*	15	33†	38†	25†	30†
	1010	811	1028	989	922	706	918	775
	64†	15†	23†	35†	21†			
	984	1189	788	698	780			
N_2 pre-treatment	14	18	37*	41*	10	8	9	
	938	920	800	790	816	701	862	
	52*	60*	71*	27	73†	24†	43†	
	767	707	1115	775	804	822	856	
	45	19	16	42†	49†			
	802	978	997	984	1024			

* Best determinations on basis of spontaneous expulsion of bubbles, or slight pressure required.
† Pupa pressed completely (to evisceration).

CO_2 alone. In Cecropia the concentration would have to be nearly 80 per cent, judged from our rough estimate of 75 μl/g for tracheal volume and SCHNEIDERMAN and WILLIAMS' finding of 57 μl/g for average burst volume.

2. THE PUPAL TRACHEAE AS A DIFFUSION PATHWAY

Because of the empirical and theoretical importance of the tracheae and spiracles in CO_2 retention, an attempt was made to measure the main anatomical parameters of the respiratory system of the *Agapema* pupa for use in future computations. Dissection was confined to abdominal spiracles and adjacent tracheal "trees", of which there are 6 functional pairs, and the assumption was made that these are anatomically and physiologically equivalent to the thoracic pair.

The stigma or frame of the external opening into the tracheal system (Fig. 2A) consists of a rigid sclerotized "filter apparatus" (F) set in a shallow depression within the oval stigmal border or peritreme (P) on the pupal surface. Actually, the two sides of the filter consist of transversely ribbed or fluted cylindrical lips which curve steeply inward on either side of a narrow slit, thus much resembling the aperture of a cowrie shell. The main slit is about 535 × 27 μ, and each of the 25 lateral spaces between the flutings on each lip is 3–5 × 15–20 μ, giving a total area for the aperture of about 5000 μ^2 (5×10^{-5} cm^2). The length of the passage through the slit (perpendicular to the body surface) is 130 μ ($1{\cdot}3 \times 10^{-2}$ cm).

Just internal to the stigmal slit is a spiracular chamber or atrium (AT, Fig. 2C), elliptical in cross-section (470 × 170 μ) and 300 μ long. At the internal end of the atrium is the spiracular valve itself (Fig. 2B; V, Fig. 2C). Since this has been

exhaustively described by BECKEL (1955) in *Hyalophora* (Cecropia), another saturniid, no details of its structure will be given except to state that the length of the valve lips is about 400 μ, that the thickness of the lips—that is, the length of the valve passage in the direction of diffusion—is about 50 μ (5×10^{-3} cm), that the

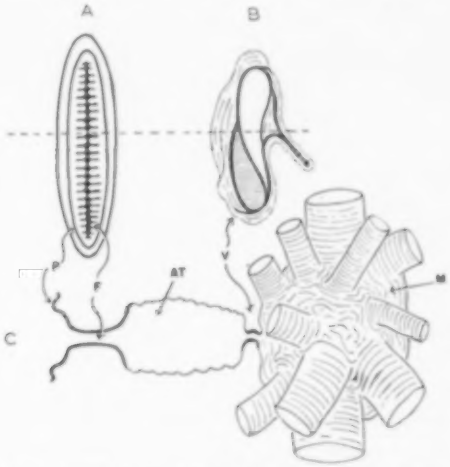

FIG. 2. Diagrams of *Agapema* spiracular apparatus. A shows an external surface view of the stigmal filter (F) within the peritreme (P). B is a view of the spiracular valve (V), in open position, as seen from the outside of the pupa looking through the atrium with peritreme and filter removed. C is a longitudinal section, taken at right angles to the long axes of filter and valve (see broken line) to indicate the restrictions in gas path at these points. AT = atrium, M = tracheal manifold.

valve appears capable of closing completely, and that by pulling upon appropriate structures in a dissection the lips can be made to separate to give an apertural area up to the order of 40,000 μ^2. In living pupae the valve can be seen to flutter open to areas estimated to be nearly 6000 μ^2, but this figure has little value because of possible foreshortening. In any case no conclusion can be drawn about normal dimensions during either burst or interburst because the valve cannot even be seen in the living pupa without an operation involving removal of most of the stigmal plate.

Immediately internal to the spiracular valve is a 720 × 535 × 355 μ chamber, which we call the "tracheal manifold" (M, Fig. 2C) because of the 17 or so large tracheal trunks given off radially to the surrounding tissues, where they arboresce profusely. Careful measurement of all the trunks issuing from the manifold gives a total cross-sectional area of the order of 38,000 μ^2 ($3{\cdot}8 \times 10^{-4}$ cm^2).

The maximum diffusion distance in the pupa, i.e. the distance from either member of a pair of spiracles of one segment to the point in that segment most remote from both spiracles, is about 5 mm (5×10^{-1} cm).

In spite of the evidence that the spiracular valves control the CO_2 burst cycle, it is necessary, before gradients or valve areas can be computed, to make certain that there is no other point in the gas transfer path at which diffusion limitation

could occur. Since the rate of total diffusive transfer of a particular gas must be the same at all levels of the path, and since the rate of oxygen uptake is known, the relative resistances of various regions of the respiratory unit consisting of a single spiracle and its associated tracheae can be obtained readily by computing the concentration gradient of O_2 necessary to maintain the known transfer rate through each anatomical step in the overall diffusion path. For these computations we use a rearrangement of the familiar Fick equation, $C_o - C_i = \frac{R_D L}{DA}$, where $C_o - C_i$ is the concentration difference (cm^3/cm^3) over the diffusion path, R_D (cm^3/sec) the rate of gas transfer, L (cm) the length of the path, D (cm^2/sec) the diffusivity of the gas in question, and A (cm^2) the cross-sectional area of the diffusion pathway.* In addition to parameters already given we need values for rate of O_2 uptake (16 μl/g/hr $= 3 \times 10^{-7}$ cm^3/sec/spiracle) and for the diffusivity of O_2 at 25° (2×10^{-1} cm^2/sec). Also we make the assumption that the total cross-sectional area of all the tracheae and branches supplying a single manifold is constant at all distances from the manifold. This assumption is in accord with measurements of KROGH (1920) and of THORPE and CRISP (1947).†

Now, for transfer through the stigmal slit,

$$C_o - C_i = \frac{R_D L}{DA} = \frac{(3 \times 10^{-7}) \times (1{\cdot}3 \times 10^{-2})}{(2 \times 10^{-1}) \times (5 \times 10^{-5})} = \frac{4 \times 10^{-9}}{10 \times 10^{-6}} = 4 \times 10^{-4} \text{ cm}^3/\text{cm}^3,$$

which is about 0·3 mm Hg in partial pressure units (negligible). A similar computation for the atrium, using $6{\cdot}2 \times 10^{-4}$ cm^2 for the cross-section computed as an ellipse, gives also a negligible concentration drop (7×10^{-5} $cm^3/cm^3 = 0{\cdot}05$ mm Hg), and for one spiracular manifold and its entire associated tracheal tree the pressure drop is less than 0·1 mm Hg, even assuming that all the oxygen travels the maximum 5 mm path to the tissues.‡ Since then, neither the stigmal plate, spiracular atrium, manifold nor tracheae offers any significant resistance to gas diffusion it follows that the spiracular valve must be the site and cause of any gas concentration fall that occurs between environment and tissue, or in the opposite direction. The actual dimensions of the limiting valve aperture will vary under different conditions and cannot be computed without knowing the gas concentration gradients involved. These will be considered in future papers.

3. BLOOD VOLUME; BODY AND TISSUE WATER CONTENT

For use in estimating CO_2 storage capacity, the partitioning of pupal water was investigated in *Agapema*. Blood was collected by cracking off the head capsule,

* We are much indebted to Dr. J. VERDUIN for instruction in the computational method.

† NUNOME (1944) reported a constantly decreasing total area beyond the main tracheae, but since he did not report the number of branches at each level, an essential though elusive datum, it is possible that sufficient account had not been taken of the enormous distal increase in numbers of collaterals.

‡ Use of the "pore-diffusion" correction factor for L (BROWN and ESCOMBE, 1900; VERDUIN, 1954) would increase the computed gradient values from 7 to 50 per cent, depending on the dimensions involved, but since the gradients would still be negligible we have omitted the corrections in the interest of simplicity.

making slits into the wings, and putting the animal head-down into a sedimentation tube graduated to 4 μl. The tube was then centrifuged briefly at low speed and the resulting weight and volume of clear, tissue-free blood were recorded. This was repeated several times until the volume of extracted blood was constant. Then the centrifugal force was increased until the gut ruptured and added its black liquid content to the blood. Centrifugation was continued until no more black liquid appeared and the tissues began to be displaced out of the pupal shell. The difference between true blood (which averaged about 33 per cent of pupal fresh weight) and total free liquid extractable by centrifugation was recorded as "gut liquid" (which averaged about 10 per cent of fresh body weight). Fresh exoskeleton, scraped free of tissues, averaged from 15 per cent to 24 per cent of total body weight depending on whether the pupa was large (1·0–1·2 g) or small (0·5–0·6 g), and contained about 60 per cent water. (Since these pupae were 7–8 months old at the time of analysis it is possible that cuticle dry weight was somewhat higher than average, as compared with total dry weight, because of loss of body water.) Fresh tissue alone ("fat") accounted for about 34 per cent of total body weight and contained about 53 per cent water.

Whole pupae dried to constant weight at 110°C averaged about 28 per cent of their live weights, indicating that an average 1 g pupa would contain 720 μl of water. Judging from the analyses given above this 720 μl should include roughly 330 μl free in the blood, 100 μl free in the gut, 180 μl in the tissues and 110 μl in the cuticle. Estimates of body CO_2 capacity are contained in Part III of this paper (Buck and Freidman, 1958).

4. INTERBURST TRACHEAL pCO_2

For two reasons the concentration of CO_2 in the tracheal system is a key datum for the understanding of CO_2 release cycles. First, as long as tracheal CO_2 concentration is not definitely known to be too high for the observed O_2/CO_2 transfer ratio during interburst to be explained on the basis of pure diffusion, the periodic intervention of a qualitatively different type of metabolism cannot be ruled out. In other words, in spite of the apparent improbabilities of a metabolic shift occurring in the presence of unchanged O_2 uptake rate and of its being synchronized with spiracular valve changes, the possibility still exists that the rate of CO_2 release is low during interburst simply because CO_2 is being fixed during this period, and high during the burst because at that time a sudden decarboxylation occurs which raises tracheal pCO_2. Second, regardless of how CO_2 is *retained* during the interburst period, the actual *escape* of that gas seemingly can involve only diffusion. Hence any quantitative description or explanation of cyclic CO_2 release will require values for both spiracular valve area and trans-spiracular CO_2 concentration difference. Either of these parameters can be computed via the Fick equation, if valve length and CO_2 release rate are known, but the other must be actually measured. Punt (1944) guessed that CO_2 might rise to 10 per cent in the tracheae of the adult bug *Triatoma* before a burst occurred, but his value was based on the unsupported assumptions that the spiracles were tightly closed during interburst

(20 min) and that the tracheae occupied 60 per cent of body volume (DEMOLL's 1928 estimate for the adult beetle *Melolontha*).

In the absence of techniques for direct analysis of tracheal gas *in situ*, we have estimated tracheal CO_2 concentration in *Agapema* by observing the direction of gas movement when the pupa equilibrates with various CO_2–N_2–O_2 mixtures in Warburg respirometers in the absence of alkali. The idea behind this method is that when the spiracles open wide, as they do at burst time, CO_2 should leave the pupa ("burst") or enter ("negative burst") depending on whether the outside pCO_2 is lower or higher, respectively, than tracheal pCO_2.* In the procedure,

TABLE 2—EFFECT OF DIFFERENT ENVIRONMENTAL CO_2 CONCENTRATIONS ON VOLUME AND DIRECTION OF GAS EXCHANGE AT TIME OF SPIRACULAR OPENING (*Agapema*)

Percentage CO_2	No. pupae	No. measurements in air	Vol. burst in air (μl/g)	No. measurements in CO_2	Vol. change in CO_2 (μl/g)	Percentage normal burst vol.
2·5	15 (3)	37*	31·6 ± 1·0	43*	+ 26·3 ± 1·8	83
5·0	16 (2)	39*	29·9 ± 2·3	55*	+ 17·4 ± 1·7	58
5·9	19	11	31·2 ± 4·5	11†	5 pos., 6 neg.	0
8·0	9	4	14	9	− 35·5 ± 2·7	118‡
9·0	9	17*	39·4 ± 3·1	9	− 79·0 ± 8·0	200
12	16 (1)	28	35·2 ± 5·0	17	− 92·2 ± 6·7	262
25	20 (4)	33	31·8 ± 2·3	24	−215 ± 8·8	677
49	18	29	27·8 ± 2·2	18	−368 ± 7·5	1320

Numbers in parentheses after number of pupae indicate number used twice (i.e. on a second day). All individuals listed as being tested in CO_2 concentrations of 8 per cent or higher gave at least one negative burst. The values for negative burst volume in 9 per cent, 12 per cent and 25 per cent CO_2 include the extra CO_2 which leaked in during the flush-burst interval. This correction was too small to be estimated accurately with 8 per cent CO_2, and with 49 per cent CO_2 the bursts occurred too soon.

* Include at least 2 measurements on almost every pupa.

† The 5 positive bursts averaged 14·4 μl/g and the 6 negative bursts 17 μl/g. Eight pupae gave no detectable burst in either direction in at least 5 hr.

‡ Assuming that a larger sampling would have shown an air burst volume close to the usual 30 μl/g.

CO_2 release rate was measured in air until (usually) two normal bursts had occurred, then the flasks were flushed with a known mixture for 5 min at a flow rate of 500 μl per min. The gas combinations were made by mixing CO_2 and air in the desired proportions via calibrated flowmeters. Because the triggering pCO_2 for spiracular opening in certain adult insects has been reported to vary with ambient pO_2 (WIGGLESWORTH, 1953; CASE, 1956), we took care to bleed into each mixture the amount of O_2 necessary to keep the total O_2 concentration at 21 per cent as monitored with a Pauling meter.

* We are indebted to Dr. H. SPECHT for originally interpreting the volumetric changes occurring with high ambient pCO_2; and to Dr. RUBERT ANDERSON for showing us how such measurements could be used to estimate tracheal pCO_2.

Table 2 summarizes the results of tests with several mixtures, and shows that the predicted transfer of CO_2 in both directions actually occurred. Another confirmation of the soundness of the experiment is the fact that the average interval between flushing and spiracular dilatation varied inversely with the CO_2 concentration of the ambient gas ($2\frac{3}{4}$ hr with 9 per cent; $2\frac{1}{2}$ hr with 12 per cent; 1 hr with 24 per cent; 35 min with 49 per cent) as would be expected if the CO_2 were leaking into the tracheal system and accelerating the rise to triggering concentration. The actual volumes of CO_2 exchanged may be slightly in error because of simultaneous exchange of other gases, but both the direct tests with 5·9 per cent CO_2 and interpolation from other concentrations (Fig. 3) indicate that tracheal CO_2 concentration at the time of spiracular opening is about 6 per cent (pCO_2 = 45 mm Hg). This value is the same as in the honey-bee (BISHOP, 1923), an insect not known to exhibit cyclic CO_2 release.

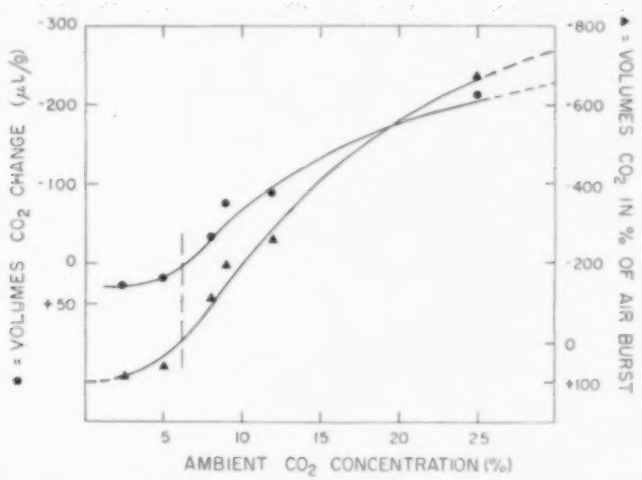

FIG. 3. Relation between ambient CO_2 concentration and volume of CO_2 moving out of (+ values on ordinates) or into (− values on ordinates) *Agapema* pupae at the time of presumed spiracular opening. Values expressed both in terms of absolute volume (left ordinate) and percentage of normal burst volume in air (right ordinate). Dashed portions of lines in upper right corner indicate course to values for 49 per cent ambient CO_2. Vertical broken line indicates normal tracheal CO_2 concentration (6 per cent) with zero gas exchange.

It should be pointed out that although the *decrease* in burst volume in 2·5 per cent and 5·0 per cent CO_2 probably resulted from changed triggering level of CO_2, in ambient concentrations above 6 per cent the valves do not constrict again, once they have dilated, hence the volume of CO_2 that enters is the amount required to fully equilibrate the blood and tissues.

We were unable to use the above technique in estimating the tracheal pCO_2 of the Cecropia pupa because the negative bursts always occurred before the end of the period of temperature equilibration after flushing.

The 6 per cent value for estimated tracheal CO_2 concentration, when considered in relation to the average tracheal volume of 60 μl, shows that not more than 3·6 μl of the average burst volume of about 30 μl could come from CO_2 initially in the gas phase. This finding thus supports the opinion of SCHNEIDERMAN and WILLIAMS in regard to the Cecropia burst. Besides indicating the need for information on the CO_2 capacity of the blood and tissue liquids—a matter considered in Part III of this paper—the fact that the CO_2 in the burst must come from two sources may have some bearing on the characteristically asymmetrical time course of CO_2 release during the burst. Thus the rate of CO_2 release typically rises almost instantaneously to a peak from which it declines much more slowly. This is shown even in Warburg records of bursts (e.g. SCHNEIDERMAN and WILLIAMS, Figs. 2, 4) but is particularly striking in PUNT's fast recordings (e.g. 1950, Figs. 3, 9, 12). Part of this skewing may be related to the sort of spiracular valve flutter observed by SCHNEIDERMAN (1956) or, conceivably, to asynchronous opening or closing among the different individual or pairs of valves. However, it would also be expected that the burst would involve an initial very rapid diffusive escape of the CO_2 already in the tracheal gas phase, followed by a slower escape of CO_2 from blood and tissues. Such a differentiation between the escape rates of gas phase and dissolved phase CO_2, *apparently before the spiracles constrict*, is perhaps shown in Fig. 1 of PUNT, PARSER and KUCHLEIN (1957). Evidence that release of CO_2 from body liquids is by no means instantaneous is also seen in our observation that even after equilibration with CO_2 concentrations as low as 12 per cent, the giving back of the extra CO_2, upon return of the pupa to air, was detectable for 30 min. Similarly, the "purging" of body CO_2 which occurs when the valves are forced to dilate widely by putting the pupa in N_2 (BUCK and KEISTER, 1955) lasted an average of 32 min (17 pupae) as compared with 12 min for normal bursts in air.

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5. CO_2 RELEASE RATE DURING THE INTERBURST PERIOD

If a near equilibrium between gas in tissue liquid and tracheal gas exists throughout the interburst period, the concentration of CO_2 in the gas phase should increase as the metabolic CO_2 is impounded and the concentration of free gas and its combination products with water increase in the liquid phase. Quite aside from changes due to possible spiracular flutter, therefore, the rate of CO_2 leakage should rise throughout the interburst period. Curiously enough no such rise was observed by SCHNEIDERMAN and WILLIAMS (1955) nor is any perceptible in our individual Warburg records (e.g. BUCK and KEISTER, 1955, Fig. 1).* This could be due to a progressive diminution in spiracular valve area during interburst. Since, however, it is difficult to imagine either a reason why the pupa should keep the *rate* of CO_2 release constant, or a control mechanism for accomplishing this, we investigated the alternative possibility that the rate of increase is too slight to show in individual records. By computing the net rate of gas exchange during the

* It is difficult to be certain about the baseline in PUNT's diaferometer records because of possible galvanometer drift and lack of absolute concentration calibration, but some of his records seem, disturbingly, to show no sign of an increased rate of CO_2 leakage between bursts.

three hours immediately preceding the burst in 95 cycles of 3–6 hr duration (mean = 4⅓ hr) it was shown clearly (Fig. 4) that the rate of change in volume declines as the time of the burst approaches. (It will be remembered that in the "direct" Warburg method, CO_2 release is not measured directly but is obtained as the difference between O_2 uptake measured in the presence of alkali and the net gas volume change (O_2 minus CO_2) when there is no absorption of CO_2.) The apparently accelerated rate of change just before the burst may be an artifact due to valve flutter (cf. SCHNEIDERMAN, 1956), but considered as either a straight or

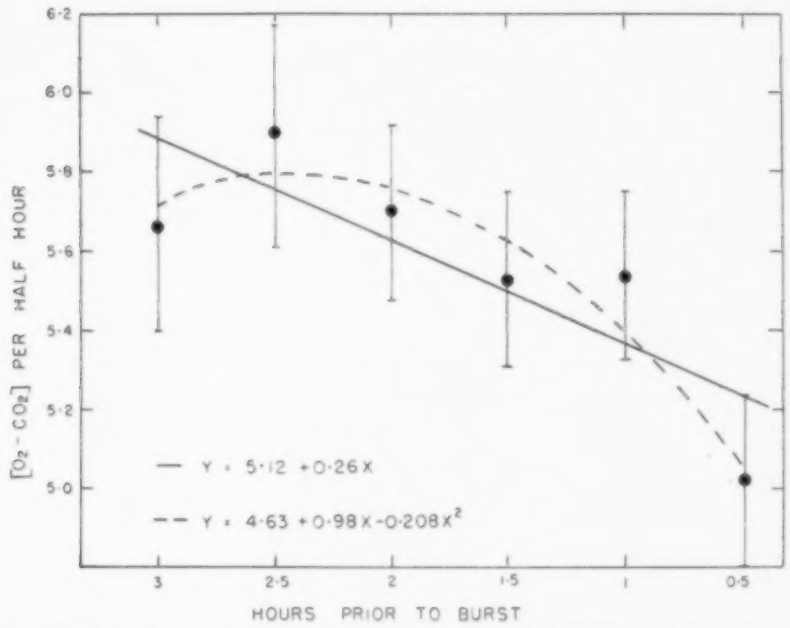

FIG. 4. Pre-burst decrease in rate of net pupal gas uptake with time, presumably reflecting an increased rate of CO_2 leakage. Cap-lines indicate standard errors.

curved line the regression is significant by F test at the 5 per cent level, and corresponds roughly to a 20 per cent decrease in net rate of gas uptake during the interburst portion of a mean cycle length of 4⅓ hr. If oxygen uptake were constant throughout, an increase in rate of CO_2 release of about 20 per cent per cycle would be indicated. Though O_2 uptake appears to be constant with time in individual records, it cannot be safely concluded that no mean change would have been detectable in the 95 records if it had been possible to measure O_2 and CO_2 exchange simultaneously. An approach to this problem was made by averaging 23 other O_2 uptake records in which it was possible to identify the time of occurrence of bursts by small but significant artifacts previously observed (BUCK and KEISTER, 1955). In these records there was an apparent decrease in mean O_2 uptake rate with

approach to burst time, but the linear regression on time was not statistically significant. It can be concluded tentatively, therefore, that the rate of CO_2 release during the interburst period does increase progressively. If we consider 6 per cent to be the triggering CO_2 concentration for the burst, the maximum range of change during the interburst period would be from 5·0 to 6·0 per cent or from 38 to 45 mm. This conclusion resolves what had previously appeared to be a contradiction to the idea that most of the metabolic CO_2 produced between bursts is impounded in the pupal buffer systems, and, together with the information discussed in section 2, reinforces the evidence that cyclic CO_2 retention in insects involves constriction of the spiracular valves.

6. RELATION OF METABOLIC RATE TO CYCLIC RESPIRATION

The prevalence and striking nature of cyclic CO_2 release in diapausing lepidopterous pupae has tended to create the impression that the phenomenon is somehow characteristically or causally connected with a low metabolic rate. This impression has been strengthened by such findings as those of ITO (1954) in which the normally non-diapausing and non-cyclic *Bombyx* pupa was converted into a burst producing type by paralysis caused by ligaturing behind the brain, and of SCHNEIDERMAN and WILLIAMS (1955) in which Cecropia pupae that had ceased to give CO_2 bursts because they had broken diapause and begun adult development were induced to resume "bursting" when the temperature (metabolic rate) was lowered. We report below (Table 3) a similar result in that *Bombyx* pupae exhibiting continuous CO_2 release at 25°C developed unmistakable CO_2 cycles at 15° and 10°, in agreement with a prediction of SCHNEIDERMAN and WILLIAMS (1955).

TABLE 3—CO_2 BURST PRODUCTION IN *Bombyx* PUPAE. ALL PUPAE 1–4 DAYS AFTER PUPATION (CHANGES GIVEN IN ARBITRARY MANOMETER UNITS, IN THE ABSENCE OF FLASK ALKALI)

Temperature	Duration of measurement (hr)	No. pupae	Range and sign of volume changes
25°	4	10	−4 to −14; no bursts
15°	5½	13	2 pupae gave bursts (+1 to +5)
15°	4½	14	10 pupae gave bursts (+2 to +11)
10°	4¾	14	7 pupae gave bursts (+2 to +8)

Since PUNT discovered cyclic CO_2 release originally in adults of *Triatoma* at rest and has since found it in adults of other species (*Locusta*, *Meloe*, *Rhodnius*, *Carabus*, *Hadrocarabus*), it cannot be argued that the phenomenon is associated with some metabolic or structural peculiarity of immature insects. Furthermore, such meagre evidence as exists gives no grounds for believing that insects in which cyclic CO_2 release has been demonstrated differ systematically in relative tracheal volume, blood volume or tracheal pCO_2 from those in which it has not been

demonstrated; and Part III of this paper shows that the same is true for CO_2 capacity. However, the adult insects mentioned above, being either lethargic (when fed) or having been studied at low temperatures (e.g. 15°C), do have inactivity in common with the lepidopterous pupae. It is accordingly possible that it is not low metabolic rate *per se* that is necessary for cyclic CO_2 release, but low metabolism in relation to O_2 supply. There could, in other words, be no CO_2 retention without valve constriction, and no sufficient valve constriction unless respiratory requirements were low. This would be in accord with our previous (1955) suggestion that interburst valve area is held to "the minimum compatible with adequate respiratory O_2 supply" in the interests of water conservation. If this hypothesis of minimal interburst valve area is valid, it might be anticipated that discontinuous respiration could be induced in species not normally showing it, by increasing the ambient O_2 concentration. This experiment has not actually been done, but confirmation in principle is offered by a Cecropia pupa reported to exhibit only continuous CO_2 release in air, but a typical cycle in 40 per cent O_2 (SCHNEIDERMAN and WILLIAMS, 1955).

In sum, it seems reasonable to guess that cyclic CO_2 release is potentially widespread, previous failure to detect it being due to (a) having more than one individual in the respirometer, (b) use of respirometers with poor time resolution, and (c) testing the insects under conditions in which a sufficiently constricted spiracular valve setting could not be sustained (for example, at too high a temperature). In this connexion, the triggering of spiracular valve activity during the cycle has been shown to have much in common with normal spiracular control in insects not known to exhibit cyclic CO_2 release (BUCK, 1957).

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EXPERIMENTELLE AUSLÖSUNG VON HÄUTUNGEN BEI DER TERMITE *KALOTERMES FLAVICOLLIS* (FABR.)*

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Abstract—Termite nymphs can be caused to moult by injecting or feeding with prothoracic gland hormone (α-Ecdyson). The induced moults lead without growth to normal nymphs and pseudergates. The moulted nymphs show no signs of progress towards imaginal differentiation and no supplementary reproductives are produced. The classification of the prothoracic gland hormone as a growth and differentiation hormone (SCHARRER, 1948, 1952) seems questionable under these circumstances.

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BEI der Termite *Kalotermes flavicollis* scheint die Produktion von Ersatzgeschlechtstieren durch Ektohormone gesteuert zu werden, welche von den funktionellen Geschlechtstieren produziert und von den Larven in der Kolonie verbreitet werden (LÜSCHER, 1956). Diese Ektohormone verhindern kompetente Larven und Nymphen daran, sich in Ersatzgeschlechtstiere umzuwandeln.

Es stellt sich nun die Frage, wie die Ektohormone in das hormonale Geschehen der Larven und Nymphen eingreifen. Da die Ersatzgeschlechtstierhäutung eine durch das Fehlen der Geschlechtstiere verfrüht ausgelöste Häutung ist (LÜSCHER, 1952a), liess sich vermuten, dass die Wirkung der Ektohormone in einer direkten oder indirekten Hemmung der Prothoraxdrüsen besteht. In diesem Falle müssten bei künstlich verfrüht ausgelösten Häutungen auch bei Anwesenheit von Geschlechtstieren Ersatzgeschlechtstiere entstehen. Mit der Isolierung des Wirkstoffs "α-Ecdyson" (Prothoraxdrüsenhormon) aus *Bombyx*-Puppen (BUTENANDT und KARLSON, 1954; KARLSON, 1956) war die Möglichkeit gegeben, diese Vermutung experimentell zu prüfen.

Nachdem sich herausgestellt hatte, dass die Injektion von gereinigten Extrakten eine sehr hohe Sterblichkeit der Termiten zur Folge hatte, wurde für Injektionsversuche das reine kristallisierte α-Ecdyson, in Insektenringer gelöst, verwendet. Mit Hilfe einer Mikroinjektionsspritze wurden 0,1 bis 1 mm^3 Hormonlösung von der Dorsalseite in das Abdomen injiziert. Eine grössere Kolonie (Herkunft: Banyuls sur Mer, Frankreich) wurde in fünf Gruppen von je 25 Larven und Nymphen gleichmässig aufgeteilt. Zu jeder Gruppe wurde ein Paar funktioneller Ersatzgeschlechtstiere zugesetzt, und jede Larve oder Nymphe einer Gruppe erhielt die gleiche Dosis Hormon. Die Ergebnisse des Versuchs sind in Abb. 1 und Tabelle 1 zusammengestellt.

* Durchgeführt mit Hilfe eines Forschungskredits des Schweizerischen Nationalfonds.
† Gast des Theodor-Kocher-Institutes, Bern, Januar 1957.

In einer weiteren Serie von Versuchen wurde Ecdyson an 5 mit Geschlechtstieren versehene Gruppen von Termiten verfüttert. Hierzu wurde ein gereinigter Extrakt auf Filtrierpapier aufgetrocknet verabreicht. Zwei Kontrollgruppen

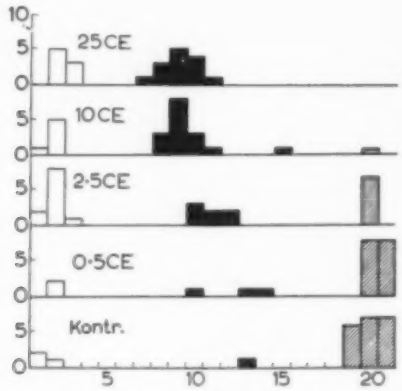

ABB. 1. Injektionsversuche mit Ecdyson. Ordinate: Anzahl Termiten; Abszisse: Tage nach Versuchsbeginn. Weiss: gestorben; schwarz: gehäutet oder während der Häutung gestorben; schraffiert: nach 20 Tagen nicht gehäutet.

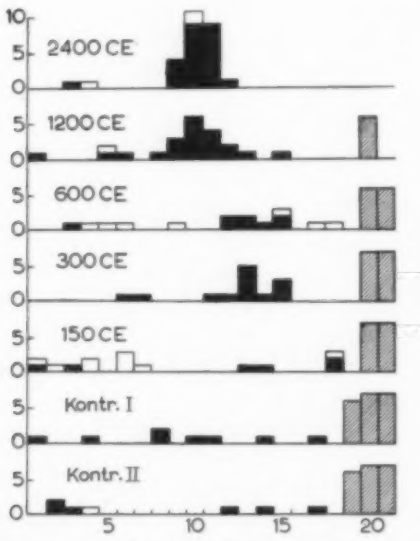

ABB. 2. Fütterungsversuche mit Ecdyson. Ordinate: Anzahl Termiten; Abszisse: Tage nach Versuchsbeginn. Weiss: gestorben; schwarz: gehäutet; schraffiert: nach 20 Tagen nicht gehäutet. Vereinzelte Ersatzgeschlechtstier-oder Imaginalhäutungen, die in Versuchs- und Kontrollserien in ungefähr gleicher Zahl auftraten, wurden in dieser Darstellung nicht berücksichtigt.

erhielten unbehandeltes Filtrierpapier. Die Fütterungsversuche sind in Abb. 2 und Tabelle 1 zusammengestellt.

Sowohl durch Injektion als auch durch Verfütterung können Häutungen ausgelöst werden. Die stärksten Dosen führten in beiden Fällen zu einer 100 prozentigen Wirkung. Bei Injektion genügen 2,5 CE (Calliphora-Einheiten) um 50 Prozent Häutungen auszulösen, während 0,5 CE eine gerade noch nachweisbare Wirkung ergeben. Bei Verfütterung wird eine etwas höhere Dosis benötigt. Bei

TABELLE 1—HÄUTUNGSAUSLÖSUNG BEI TERMITEN DURCH ECDYSON

Behandlung und Hormondosis	Gestorben	Häutungen*		
		Anzahl	% der Überlebenden	Tage nach Versuchsbeginn (Durchschnitt)
Injektion 25 CE in 1 mm^3	8	14	100	9,1
Injektion 10 CE in 0,4 mm^3	6	16	94	9,5
Injektion 2,5 CE in 0,5 mm^3	11	7	50	10,9
Injektion 0,5 CE in 0,1 mm^3	2	3	16	12,3
Injektion Ringer 1 mm^3	3	1	5	—
Fütterung 2400 CE	3	23	100	10,3
Fütterung 1200 CE	1	18	75	10,6
Fütterung 600 CE	5	8	40	13,9
Fütterung 300 CE	0	11	44	13,4
Fütterung 150 CE	8	4	22	15,7
Fütterung Filtrierpapier (Kontrolle) 1	0	6	23	—
Fütterung Filtrierpapier (Kontrolle) 2	1	3	13	—

* Die Häutungen vor dem siebenten Tag sind nicht berücksichtigt, da sie nicht auf die Wirkung des Hormons zurückzuführen sind.

300 CE pro Gruppe oder 10 CE pro Termite ist die Wirkung auf die Häutungen noch deutlich. Die Dosis, welche die einzelne Termite in diesen Versuchen aufnahm, ist natürlich nicht abzuschätzen. Sie liegt jedenfalls weit unter dem angegebenen Wert, da nur ein kleiner Teil des verabreichten, mit Hormon getränkten Filtrierpapiers gefressen wurde.

Es besteht eine Beziehung zwischen der Dosis und der durchschnittlich für die Häutung benötigten Zeit (Tabelle 1). Niedrigere Dosen lösen die Häutung etwas später aus.

Die erste sichtbare Reaktion auf das Prothoraxdrüsenhormon ist die Entleerung des Enddarms, nach welcher das Abdomen der Termiten hell erscheint. Sie erfolgt bei starken Dosen durchwegs während des ersten Tages, bei schwachen Dosen am zweiten oder dritten Tag nach der Verabreichung des Hormons. Die Häutung findet normalerweise durchschnittlich 7 Tage (LÜSCHER, 1952a), nach Ecdysoninjektion jedoch 8–10 Tage nach der Darmentleerung statt. Der normale Häutungsprozess scheint also rascher abzulaufen als der durch ein malige Ecdyson-Gaben experimentell ausgelöste.

Die durch Ecdyson induzierte Häutung ist auch der normalen Ersatzgeschlechtstierhäutung nicht vergleichbar, da diese noch rascher, schon durchschnittlich 5,4 Tage nach der Darmentleerung erfolgt (LÜSCHER, 1952a). Es ist deshalb anzunehmen, dass die Ektohormone der Geschlechtstiere nicht nur die Prothoraxdrüsen hemmen, sondern auch die Reaktionsfähigkeit der Epidermis herabsetzen.

Entgegen den Erwartungen entstanden bei den verfrüht ausgelösten Häutungen keine Ersatzgeschlechtstiere. Das Prothoraxdrüsenhormon löst also lediglich Häutungen aus, hat jedoch keinen Einfluss auf die Art der Häutung. Die entstandenen Larven und Nymphen zeigten auch keinerlei imaginale Differenzierungen und die Grösse der Oozyten in den Ovarien blieb unverändert. Die meisten behandelten Larven waren Pseudergaten, bei denen normalerweise bei der Häutung kein Wachstum erfolgt (LÜSCHER, 1952b). Auch die Versuchstiere zeigten kein Wachstum.

Das Prothoraxdrüsenhormon hat also bei der Termite *Kalotermes* keinen Einfluss auf Wachstums- und Differenzierungsprozesse. Es wirkt lediglich als Häutungshormon. Damit werden die Befunde von HALBWACHS und JOLY (1957) bestätigt, die nach Implantation von Ventraldrüsen (= Prothoraxdrüsen) bei *Locusta* verfrühte Häutungen auslösen konnten, ohne dass Wachstums- und Differenzierungsprozesse beeinflusst wurden. Sollten sich solche Beispiele mehren, so müsste die für das Prothoraxdrüsenhormon gebräuchliche Bezeichnung "Wachstums- und Differenzierungshormon" (Growth and differentiation hormone; SCHARRER, 1948, 1952) fallen gelassen werden.

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ACTION DE LA GLANDE VENTRALE SUR LE DÉVELOPPEMENT OVARIEN DE *LOCUSTA MIGRATORIA* L. (ORTHOPTERA)

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Abstract—The removal of the ventral glands which either delays or stops moulting, only partly inhibits the development of the ovaries. Among the "larves permanentes" obtained in this way the ovaries stay between those of the older larvae near metamorphosis and the ripened ovaries of adult animals.

INTRODUCTION

Dans deux publications précédentes (Strich, 1953, 1954) nous avons établi l'existence de la glande ventrale chez *Locusta migratoria* et étudié son cycle. Par ablation de cette glande sur des stades IV et V nous avons obtenu des animaux incapables d'effectuer leurs dernières mues et que nous avons appelé stades IV ou V permanents.

En observant leur comportement, nous avons constaté que ces animaux quoiqu'étant arrivés à l'âge d'adultes, ne copulaient jamais entre eux. D'où l'idée que leur développement sexuel a du être arrêté par l'ablation de la glande ventrale. Nous avons donc étudié l'appareil genital; premièrement de témoins et deuxièmement des stades IV et V permanents. L'étude des mâles n'ayant fourni que des renseignements peu précis nous nous sommes uniquement occupés de l'ovaire.

MATÉRIEL ET TECHNIQUES

Ce travail a porté sur *Locusta migratoria* L. Ces animaux sont élevés dans des cages où l'on maintient une forte densité de population. On place dans la cage des animaux adultes un pondoir rempli de sable humide dans lequel la femelle dépose ses oeufs.

L'ovaire est disséqué sous Ringer, puis à l'aide de pinces fines on dégage une vingtaine d'ovarioles des trachées qui les entourent. Chaque ovariole est sectionné à sa base et dépouillé de la membrane péritonéale qui l'entoure, laissant ainsi apparaître les ovocytes. On mesure ensuite au micromètre la longueur des trois premiers ovocytes de ces vingt ovarioles.

ÉTUDE DES TÉMOINS

L'ovaire est localisé à la partie dorsale de l'abdomen. Il est plaqué sur le tube digestif et entouré de trachées et de tissu adipeux.

Chaque ovaire est constitué par une quarantaine d'ovarioles qui aboutissent dans l'oviducte. Les ovarioles sont formés d'une chambre terminale et d'un tube

ovarien. Chaque ovariole contient une douzaine d'ovocytes qu'on numérote I, II, III etc. à partir de celui qui est le plus près de l'oviducte en remontant vers la chambre terminale. Quand les œufs sont mûrs, ils s'accumulent dans l'oviducte puis sont pondus.

La croissance des ovocytes, très lente et régulière chez les larves s'accélère fortement chez l'imago âgé de 8 à 15 jours. Le tableau ci-dessous donne une idée de cette croissance.

TABLEAU 1

Stade	Age à l'interieur du stade (jours)	Longueur des ovocytes (mm) I	II	III	Observations
IV	1	0,181	—	—	
V	3	0,271	0,217	—	
	5	0,406	0,303	0,242	
	7	0,488	0,353	0,291	
	12	0,615	0,455	0,353	
Imago	1	0,670	—	—	Début dépôt vitellus dans I
	6	0,750	0,540	0,459	Début formation du mucus
	10	—	—	—	Existence vitellus jaune dans I
	12	3,400	—	—	
	16	7,000	—	—	Œufs mûrs

Stade IV. Croissance lente et régulière qui fait passer les ovocytes I de environ 0,18 à 0,27 mm en une huitaine de jours que dure ce stade. Aucune trace de formation de vitellus n'est visible.

Stade V. La croissance se poursuit sans changement appréciable, la tàille des ovocytes I passant d'environe 0,27 à 0,67 mm en une douzaine de jours. L'aspect des ovocytes change peu et aucune trace de vitellus n'est encore visible.

Stade adulte. Les premières traces de vitellus apparaissent dans les ovocytes I dès le début du stade adulte, le dépot de cette substance s'accélère rapidement et dès le dixième jour l'ovocyte prend la couleur jaunâtre des œufs mûrs. Les oviductes commencent à se remplir de mucus à partir du sixième jour. Enfin, la croissance des ovocytes, sans marquer de discontinuité s'accélère rapidement surtout à partir du septième au huitième jour. Ils atteignent leur taille maxima qui est de 7 mm à 7,5 mm à 16 jours environ. C'est alors que mûrs ils tombent dans l'oviducte.

Chez un adulte de 16 jours nous distinguons deux sortes d'ovarioles: les deux tiers d'entre eux ont un ovocyte I contenant un œuf en formation prêt à être pondu, dont la longueur moyenne est de 7,2 mm et un tiers ont un ovocyte I blanc dont la longueur moyenne est de 1,8 mm, ils ont un corpus luteum à leur base. Ce sont vraissemblablement des ovarioles venant d'expulser un œuf.

A partir de cet âge, nous retrouverons, quelque soit l'âge de la bête étudiée, toujours ces deux sortes d'ovarioles en proportions plus ou moins variables.

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A 18 jours les oviductes sont pleins d'œufs.
Ces résultats sont traduits sur le graphique (Fig. 1).

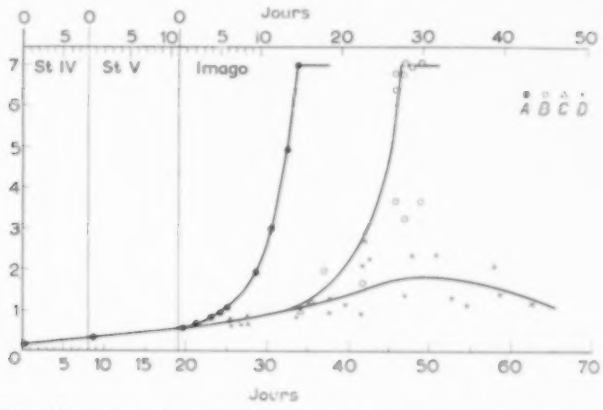

FIG. 1. Graphique de croissance des ovocytes chez *Locusta migratoria*. En abscisse, les temps en jours, l'échelle du bas est comptée à partir de la troisième mue, celle du haut est comptée à partir du début de chaque stade. En ordonnée longueur moyenne des ovocytes I de chaque animal étudié. A, animaux témoins. B, animaux privés de glandes ventrales au stade V mais néanmoins parvenus à l'état adulte. C, stades IV permanents obtenus par ablation de la glande ventrale au stade IV. D, stades V permanents obtenus par ablation de la glande ventrale au stade IV ou V.

J'ai essayé de vérifier l'échelonnement des pontes successives. Pour cela j'ai isolé des couples de *Locusta* au moment de la cinquième mue. Malheureusement l'isolement des femelles produit une mortalité précoce et élevée ainsi qu'une importante perturbation de la ponte. L'échelonnement de ces dernières a du être calculé de manière indirecte.

L'étude des ovaires de femelles fécondes élevées en groupe montre que deux générations d'ovocytes successives ont environ 6 jours de décallage (un ovaire contient simultanément des ovocytes I de 7,2 mm prêts à être pondus et des ovarioles chez qui l'ovocyte I mesure environ 1,8 mm). Dans les meilleures conditions, les pontes d'une même femelle doivent donc se succéder à environ une semaine d'intervalle.

ÉTUDE DES OPÉRÉS "PARTIELS"

Nous appelons opérés partiels des animaux ayant subi une ablation de la glande ventrale n'ayant pas supprimé la mue. Dans ce cas la métamorphose s'est effectuée avec plus ou moins de retard. L'étude de l'ovaire chez de tels animaux nous a donné les résultats suivants.

Stade IV. L'ablation effectuée sur des animaux encore au stade IV (qui ont ultérieurement accompli leurs quatrième et cinquième mues et sont donc parvenues à l'état adulte) est sans aucun effet sur l'ovaire qui est identique à celui des témoins et se développe au même rythme.

Stade V jeune. Ablations pratiquées sur des animaux juste parvenus au stade V et ayant effectué leur cinquième mue avec un retard plus ou moins considérable. On constate que le développement ovarien subit une perturbation équivalente à celle du développement somatique: leurs ovaires se développent et finissent par contenir des œufs mûrs de taille et d'aspect normal; mais avec un retard souvent assez considérable qui est de l'ordre de 12 à 15 jours.

Stade V vieux. Ablations pratiquées sur des animaux du stade V âgés de plus de 6 jours au moment de l'opération. Il est impossible de savoir si l'opération a été totale ou partielle, ce genre d'intervention étant sans action sur la mue. On constate que ce type d'intervention n'agit pas non plus sur l'ovaire dont le développement et le fonctionnement sont parfaitement normaux.

ÉTUDE DES OPÉRÉS "TOTAUX"

(1) *Ablation pratiquée sur le stade IV*

Deux cas peuvent se produire; ou bien l'ablation est faite assez précocement pour que la glande ventrale n'ait pas encore sécrété d'hormone et l'animal ne muera jamais, c'est un stade IV permanent, ou bien il y a déja assez d'hormone dans le sang pour assurer la quatrième mue et c'est seulement la cinquième qui se trouve empêchée, on a alors un stade V permanent équivalent à celui qu'on peut obtenir par une ablation de glande ventrale au début du stade V. Étant donné la brièveté et l'irrégularité du stade IV, l'opération d'ablation ne pouvant être pratiquée qu'au voisinage de la période critique il n'est pas possible d'obtenir à coup sûr et à volonté soit des stades IV soit des stades V permanents. En fait on obtient une majorité de V permanents et quelques rares stades IV.

Stades IV permanents. Nous n'avons obtenu que deux femelles bloquées au stade IV et ayant atteint l'âge où les témoins sont sexuellement mûrs et pondent.

L'aspect des ovaires était très particulier mais tout à fait analogue à celui des stades V permanents qui vont être décrits ci-dessous, leur développement se trouvait toutefois bloqué à un stade encore plus précoce. La longueur moyenne des ovocytes I était de 1,2 mm (contre 0,2 pour un témoin en fin de stade IV et 7,0 pour un témoin du même âge).

Stades V permanents. Les stades V permanents beaucoup plus nombreux que les précédents ont pu être étudiés en détail. Pendant les premiers jours suivant l'intervention, la croissance des ovocytes reste normale, ce n'est guère qu'une quinzaine de jours plus tard, alors que les témoins sont déja parvenus à l'état adulte depuis 4 ou 5 jours, que l'on constate une accélération de la croissance ovocytaire des témoins, alors que les opérés poursuivent la leur à un rythme faible. Chez les opérés la croissance ovocytaire se poursuit pendant 30 à 40 jours pour atteindre la valeur de 1,8 mm (pour la longueur de l'ovocyte I). A partir de ce moment la taille moyenne des ovocytes I tend à diminuer lentement par suite des phénomènes de dégénérescence qui éliminent progressivement les plus grands d'entre eux.

L'étude de l'ovaire nous montre une dégénérescence plus ou moins poussée de cet organe. Il est de la taille de celui d'un adulte de 12 jours environ et quelque

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soit l'âge du stade V permanent il n'a jamais dépassé ce volume. La forme des ovarioles est nettement différente de celle d'ovarioles normaux. Ils sont recourbés, tordus. L'ovocyte I contient du vitellus jaune. Il semble qu'il y ait début de formation d'œufs mais ces derniers ont une forme conique au lieu d'avoir la forme d'ellipsoïdes. Je n'ai jamais vu d'œufs dans les oviductes. La longueur moyenne des ovocytes I est chez ces animaux de 1,8 mm (contre 0,71 chez les témoins du stade V les plus âgés et 7,0 chez les images du même âge absolu).

(2) *Ablations pratiquées sur le stade V jeune*

Cette opération a des effets identiques à l'ablation sur le stade IV trop âgé. Les stades V permanents obtenus par l'une ou l'autre méthode sont en tous points identiques, tant du point de vue de l'aspect de l'ovaire que de ses dimensions. Les ovocytes I mesurent en moyenne ici aussi 1,8 mm.

La Fig. 1 et le tableau ci-dessous résument ces résultats expérimentaux: la deuxième colonne indique l'âge de l'animal à l'intérieur de son stade, c'est-à-dire le nombre de jours écoulés depuis la mue précédente, la quatrième colonne indique son âge absolu, c'est-à-dire le nombre de jours écoulés depuis la troisième mue (qui est le dernier évènement notable survenant avant le début de l'expérimentation).

TABLEAU 2

Stade	Age dans le stade (jours)	Longueur ovocyte I (mm)	Age à partir 3ème mue (jours)	Nombre d'individus
IV	0	0,17	0	—
,,	8	0,28	8	—
Permanents	35	1,20	35	2
V	0	0,28	8	—
,,	12	0,70	20	—
Permanents	47	1,80	55	14
Imago	0	0,71	20	—
,,	10	1,50	30	—
,,	15	7,00	35	—

Les valeurs affectées du signe—dans la cinquième colonne ont été calculées à partir de la courbe de croissance des ovaires des animaux témoins établie à partir de l'étude de 10 individus.

CONCLUSIONS

Les conclusions que nous pouvons tirer de l'étude expérimentale dont nous venons d'exposer les résultats sont les suivantes:

(1) Lorsque l'ablation de la glande ventrale n'a été que partielle les animaux subissent un retard dans la mue et un retard analogue dans le développement de l'ovaire qui finit toutefois par être complet.

(2) Les animaux privés de glande ventrale au stade IV ou V dont la mue a été empêchée ne développent jamais complètement leurs ovaires mais cependant

dépassent très largement le degré de développement ovarien des jeunes imagos témoins.

La maturité ovarienne semble donc indissociable de la cinquième mue et des transformations qui l'accompagnent; mais il ne nous a pas été possible jusqu'ici de déterminer parmi les processus complexes mis en jeu quel est l'agent contrôlant l'ovaire et pourquoi le blocage de celui-ci se fait à un degré de développement aussi avancé.

SUMMARY

The removal of the ventral glands of *Locusta migratoria* results either in a delay of the moult which follows the operation (when the ablation has only been partial), or the final suppression of moulting (if the ablation has been total).

The ovaries of females thus operated on, show that the operations which have been followed by a delay of moulting bring forth a delay of the ripening of the ovaries varying between 10 and 15 days; but these ovaries are quite normal and finally produce normal, ripened eggs.

On the contrary, the operations that stopped moulting completely, by changing the animals into "permanent larvae", bring with them a complete but tardy arrest of the development of the ovaries 30–35 days after the operation. These ovaries show a degree of development approximatively equal to that of 10 days old adults. Even more, they show a good many abnormal oöcytes and some which have degenerated.

Among controls, the average length of the twenty biggest oöcytes is 0·70 mm at the end of stage V a few hours before metamorphosis. Ten days after metamorphosis the average length is 1·50 mm, and 16 days after metamorphosis it is 7·20 mm and the oöcytes are laid. By "permanent stage V" the average length of the twenty biggest oöcytes reaches 1·80 mm about 30 days after the controls have undergone metamorphosis, and then decreases slowly.

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THE EFFECT OF TEMPERATURE VARIATION ON QUEEN OVIPOSITION AND COLONY FOUNDATION IN *MYRMICA*

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Abstract—Experiments show that high midsummer temperatures are not the cause of the two seasonal peaks of egg accumulation in *Myrmica*. Temperature alterations in queen-worker colony fragments cause immediate increases or decreases in the number of eggs accumulating, but numerical adjustment occurs after 14 days; egg numbers accumulating then becoming comparable to those before the temperature change. There is a compensatory adjustment of egg size in *M. laevinodis*. Egg production by solitary queens declines rapidly and is followed by the death of the queen. These results are discussed in relation to caste determination and colony foundation in ants.

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INTRODUCTION

THE present study was undertaken in the Zoology Department at Glasgow University during the years 1951–1954. Material was collected in several localities in south-west Scotland.

The species used in the course of this investigation were *Myrmica rubra microgyna* and *Myrmica laevinodis* Nyl. There has been considerable taxonomic revision of these species as originally described. *Myrmica rubra* L. was divided by NYLANDER (1846) into three forms. Successive reinvestigations as undertaken by FOREL (1874), DONISTHORPE (1927), and SANTSCHI (1931) are summarized by BRIAN and BRIAN (1949, 1955), who have shown the necessity for subdivision of the species *M. rubra* L. into the subspecies named above. The nomenclature and classification used by BRIAN and BRIAN has been followed throughout this report. There remains considerable doubt as to the exact taxonomic status of the species which have been used by previous workers.

BRIAN (1951) has described the summer population changes of colony components in *Myrmica*, and has shown that there are two periods in the year when eggs are more abundant than at other times. Such bimodal distributions of egg numbers are also recorded by LUBBOCK (1892) for *Myrmica laevinodis* and by TALBOT (1945) for *Myrmica schenki*. As BRIAN (1951) points out, this need not imply periodic oviposition rates, as a constant oviposition rate could be masked by the eating of eggs by larvae. In only one case, however, was he able to demonstrate a complete interaction between larval egg eating and egg frequency bimodality, and it appears that periodic oviposition rates must be assumed in other cases.

BRIAN (personal communication) has shown that there is no critical qualitative difference between eggs produced at these two peaks, as far as larval growth and

development towards dormancy or non-dormancy is concerned. This has been experimentally confirmed by the present author. Non-dormant larvae are, in nature, derived from eggs accumulated during June, whereas larvae produced from eggs accumulated during August normally become dormant. There is then no critical change in queen-laid eggs during the year (detectable by this means) which can be correlated with the two peaks of egg production.

The experiments described below were designed to investigate whether high midsummer temperatures affected queen oviposition to cause peaks of egg abundance.

METHODS AND RESULTS

Temperature alone was chosen for investigation. In the regulated social environment of the ant nest, microclimatic variation is probably small. Nevertheless, if any physical factor does vary significantly within these nests it is likely to be the temperature (D. A. MUIR, personal communication).

It is convenient to define at this point the terms and symbols which will be used hereafter.

The total number of eggs produced by a queen per unit time $= T_q$
The total number of eggs produced by a group of workers per unit time $= T_w$
The total number of eggs lost by being eaten by a queen per unit time $= L_q$
The total number of eggs lost by being eaten by a group of workers per unit time $= L_w$

The effective oviposition rate of a group of queens and/or workers is then the number of eggs surviving per unit time, i.e. from census to census, which can be defined as the egg input per unit time, or E. The suffix x may be used with E to denote an unknown or variable relationship of E. Specific relationships of E may be denoted by a series of suffixes as follows:

(1) In a queen worker colony from which periodic egg withdrawals are made before the onset of eclosion (three weeks at 25° C), E may be characterized by the suffixes qw:—

$$E_{qw} = T_q + T_w - L_{qw}.$$

(2) In a colony consisting of a single queen, and from which weekly egg withdrawals are made, E may be characterized by the suffix q.

$$E_q = T_q - L_q.$$

Three experiments are described. In these, queens have been cultured alone or in company with workers. These colony components have been subjected to changes of temperature within their natural range (as known from observations in the west of Scotland during the months from March to September). No larvae have been used in these experiments, and eggs have always been removed prior to eclosion.

Experiment 1

Six queens of *Myrmica rubra microgyna* were collected in early spring (February and March) before egg laying started. The queens were then kept at 22° C for about 15 days until egg laying began. They were then incubated in six separate tubes in darkness, each supplied with ample sugar and protein, and containing also a damp cotton-wool plug. (This is the normal rearing technique used throughout these and many other experiments, and has been shown to be efficient.) Three cultures were incubated at 20° C and three at 25° C. The groups of three cultures were transposed from one incubation temperature to the other after 2 weeks. Eggs were removed from the cultures every week, counted, and discarded. Results thus represented the queen input per unit time, E_q. The results of experiment 1 are shown graphically in Fig. 1. In view of the close correspondence between the three replicates of each set, the results have been averaged. The time of the temperature change is indicated on the graph by an arrow. Arrows are used in subsequent graphs to show where a temperature transposition has occurred.

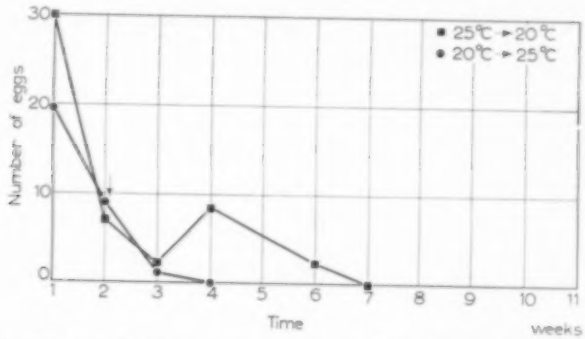

Fig. 1. The egg input of 2 groups, each of 3 small solitary queens of *M. rubra microgyna*, is shown as the average of the 3 replicates. All queens died within a week of the cessation of oviposition. Temperature change is arrowed.

Egg input was greatest during the first week of the experiment and the second week of actual oviposition. Egg accumulation in all cultures ceased within 7 weeks and was followed at once by the death of the queen. Solitary queens at 20° C lived longer than those at 25° C and achieved a greater total effective egg production throughout this period. (Fig. 1, death follows where the egg input falls to zero.) Post-mortem examinations showed that fat body in these queens was very small.

Experiment 2

Six queens of *M. rubra microgyna*, similar in size to those used in experiment 1, were placed in tubes as described previously and incubated, three at 25° C and three at 20° C. In this experiment, however, three workers were included with each queen. The colonies were changed from one temperature to the other after 14 days. The results are shown graphically in Figs. 2 and 3. Each line represents the average of the three replicates, the close agreement between all three justifying this procedure. No queens died during the 11 weeks of the experiment.

Experiment 3

This experiment was carried out using queens and workers of *Myrmica laevinodis*. Since it was desirable that all the queens and workers for this experiment should be as

homogeneous as possible in respect of both genetical constitution and previous environmental treatment, it was necessary to use a different species of ant. Thirty queens were used, the total number of queens in one nest which was regionally very isolated. They

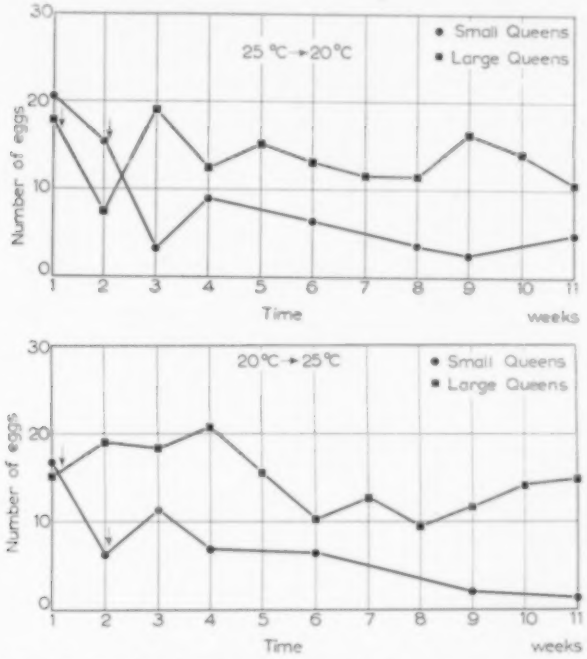

FIG. 2 and FIG. 3. Results of experiments 2 and 3. The egg input of groups of workers with a queen taken from the averaged value of 3 replicates of each temperature treatment in experiment 2, and 5 replicates of each temperature treatment in experiment 3. Small queens—*M. rubra microgyna* (experiment 2). Large queens—*M. laevinodis* (experiment 3). Temperature change is arrowed.

represent, therefore, material as genetically uniform as is practicable, with a species where pleometrosis is combined with the ability to increase queen numbers by recovery from the region round the nest of fertilized queens of their own and, presumably, other colonies. The queens were separated from the workers in the colony, and divided at random into 10 groups of 3. The separation was carried out while the queens and workers were narcotized by carbon dioxide in order to avoid the selection of more active queens first and less active ones afterwards. Carbon dioxide was not used subsequently in this experiment. Experiments show that deleterious effects are produced by repeatedly exposing workers to the gas (WEIR, 1958).

Each group of 3 queens was given 12 workers, similarly randomized, from the original colony of several thousand workers. Five of the groups were then incubated at 25° C and 5 at 20° C. After 1 week at the appropriate incubation temperature all 5 cultures of each series were changed to the alternative temperature. The original colony had been collected in the field in early April when a few eggs were present in the nest. Separation of the queens was not carried out until after a week to allow removal of injured individuals. The E_{qw} of approximately the first 10 days was therefore lost. Variation between the

replicates at each weekly egg census will be considered elsewhere. Results of each set of 5 replicates have been averaged as before, and have then been divided by 3 in order to allow direct comparison with experiment 2 on the basis of single queens. The results are shown graphically in Figs. 2 and 3.

There is no significant (F test: $P>10\%$) difference between E_{eg} at the two temperatures. The different temperatures have a measurable effect on the size of the eggs produced. In experiment 3 the length of all eggs counted was measured and the mean value found for each replicate at every census. No significant variation was detected among the group of 5 replicates at one temperature or among the 5 replicates at the other temperature. Significant differences were detected between the two temperature groups. Statistical analysis (F test: $P<1\%$) shows that the eggs produced at 25° C were significantly smaller than those produced at 20° C. Because there was no significant variation among the replicates at either temperature, the two groups of 5 replicates have each been averaged to simplify the graph (Fig. 4).

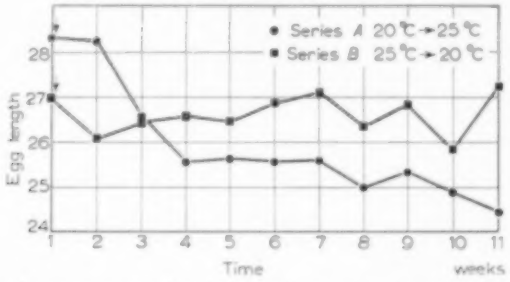

Fig. 4. Variation in average egg size in experiment 3 is shown as the average of the 5 replicates of each temperature treatment. Temperature change is arrowed.

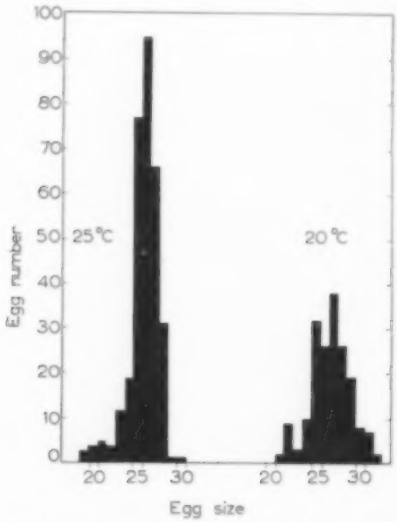

Fig. 5. Shows the differences between frequency distribution of egg sizes at two temperatures in experiment 3. There is a lower mean value at 25° C, and the degree of skewness of the distributions is different. (1 unit = 0·0232 mm.)

There is a partial overlap of the egg size range at any one census, between the summed replicates. Two frequency distributions for a typical census are shown in Fig. 5. In addition to the differences in the means of the distributions, there is a difference in the type of distribution and the degree of skewness of the distributions. Reverting to Fig. 4, it is apparent that there is a steady decline in the average size of the eggs produced at 25° C. It should be noted by comparison with Figs. 2 and 3 that the time of the alteration in average egg size shown by the 5 replicates which were changed from 20° C to 25° C corresponds to the time of readjustment of the total effective egg production to a value approximating to that before the temperature change. No striking differential viability has been detected between the two size ranges of eggs.

The effect of temperature changes on E_q or E_{qw} in experiments 1 and 2 is as follows.

If there is a temperature rise there is an immediate rise in E_{qw}.

If there is a fall in temperature there is an immediate fall in E_{qw}.

E_q continues to fall after both temperature alterations.

In experiments 2 and 3, E_{qw} stabilizes after 14 days at a value related to that before the temperature change. Thus compensation for the temperature change is achieved. While numerical readjustment of E_{qw} is achieved this does not imply that T_q is the same. L_w and L_q remain unestimated throughout the experiment, and L_w can be shown to be significantly large under certain conditions, while T_w is also unestimated.

In the case of experiment 1, a comparable effect is seen in the temporary increase in E_q in all cultures at 20° C 14 days after the temperature change.

Physiological adjustment is incomplete in at least *M. laevinodis* (experiment 3) where egg size falls as temperature rises.

DISCUSSION

Major differences between these experiments and those of BRIAN (1951) which have to be considered in comparing results are, in the present experiments: absence of environmental fluctuation, repeated removal of eggs, use of a different type of nest, and the use in some experiments of different species of *Myrmica*. In this connexion the rapid attainment of maximal E_q, maximal E_{qw}, and maximal average egg size during the first week of all experiments where these were measured is significant. The initial maxima may be due to intrinsic socio-ecological causes and may occur in nature. On this point there is no evidence, but it is possible that they are caused by the change from conditions in the field to those in the laboratory.

There is no evidence to suggest that high midsummer temperatures can account directly for the occurrence in nature of two peaks of egg abundance. On the contrary, the presence of a mechanism of numerical stabilization which, at least in some cases, is coupled with a mechanism of egg size adjustment shows that colonial sociology is adapted to circumvent or utilize the effects of high midsummer temperatures.

Seasonal egg differences have been shown to control caste determination in *Formica rufa rufo-pratensis minor* Goss. (GOSSWALD and BIER, 1954). The same

workers showed that caste determination in *Formica rufa pratensis* Retz. was controlled by trophogenic worker cycles. Caste determination in *Myrmica* has been shown (Brian, 1955a, 1955b, 1956) to be trophogenic also. Nevertheless, the variation of queen-egg size with temperature in *Myrmica* shows a possible origin of the annual cycles of blastogenic caste determination achieved by the more highly evolved *Formica rufa rufo-pratensis minor*.

Brian and Brian (1949, 1955) have discussed the problem of colony foundation in *Myrmica*. From Brian's experiments (1951) on *M. laevinodis* it appears that the solitary queen must be able to survive for about 10 weeks in the field. This 10-week period does not include the preliminary period of overwinter hibernation. During this 10-week period the solitary queen has to:

(1) Lay eggs, some of which become larvae.
(2) Survive until the eggs hatch.
(3) Feed the larvae either on allochthonous food procured by the expenditure of her own energy reserves, or on autochthonous food (eggs or glandular secretions) by the utilization of her stored food material.
(4) Survive long enough to allow pupation and worker emergence.

When the workers render allochthonous food accessible to the queen, the colony may be considered to be successfully established. The corresponding time in the laboratory at 25° C may be in the order of 6 weeks (Brian, 1954). Further information on this subject can be gained from comparison of experiments 1, 2, and 3 where there is:

(a) Increased queen survival in the presence of workers. The considerable E_{qw} over the 11 weeks of experiment 2 shows that the solitary queens of experiment 1 did not die purely as a result of adverse environmental conditions. It appears that they died because the queen by itself cannot survive in an environment which, were workers present, would represent suitable laboratory conditions for culture.

(b) A significant and large increase in E_x as a result of the transformation from E_q to E_{qw} between experiments 1 and 2, in spite of the generally accepted belief that T_w is small compared to T_q. The increase is effective both by increasing the total of eggs produced during 11 weeks, and by increasing the length of time during which the input of eggs continued.

(c) A steady decline in egg input throughout experiments 1 and 2. This decline is notable in two respects, namely, solitary queens (E_q) show more rapid decline than queens with workers (E_{qw}), and queens at high temperatures (within each of the above groups) show a higher rate of decline (whether E_q or E_{qw}) than queens at lower temperatures.

(d) A fall in egg input by small *M. rubra microgyna* queens throughout the experiments 1 and 2. Such a fall would correspond to an annual decrease in E_{qw} in this subspecies. This effect is not shown by the larger *M. laevinodis* queens which have a higher E_{qw} throughout the experiment (3).

(e) Exhaustion of the fat body of isolated queens (experiment 1) showing that their initial egg production is derived largely from stored reserves in the fat body

or redundant internal organs (e.g. the thoracic musculature (JANET, 1907)). Isolated queens failed to survive for the minimal 6 week period in culture at 25° C (experiment 1), probably because the energy normally expended in the several processes involved in colony establishment was utilized for the repeated production of an egg mass.

The solitary queen does not utilize environmental food in sufficient quantity to keep it alive over the critical 6 week period. The addition of workers makes this environmental food available to the queen.

The larger egg input, the absence of any apparent seasonal decline with the greater bulk of the fat body of the macrogyne—here observed in *M. laevinodis*, though at least the two latter facts are true of *M. rubra macrogyna*—show that the macrogyne may be more fitted to act as a colony foundatrix than the microgyne. The much higher frequency of occurrence in the west of Scotland of young colonies of *M. rubra macrogyna* compared with *M. rubra microgyna* appears to confirm this observation.

Similarly the numerically greater egg input of solitary queens at 20° C, and the production of larger eggs at 20° C than at 25° C by queens with workers, show that sociological efficiency, as measured by the maximal bulk of eggs (potential juvenile material) produced, is greatest at 20° C. Culture at 20° C then presents a closer approach to optimal conditions than does culture at 25° C.

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SUMMARY

(1) Experimental investigation of the effect of temperature variation on oviposition by isolated queens and small groups of queens and workers shows that there is no evidence that high midsummer temperatures directly cause the two seasonal peaks of egg abundance previously described in *Myrmica*.

(2) In queen worker colony fragments of *M. laevinodis* and *M. rubra microgyna*, temperature change from 20° C to 25° C causes increased egg accumulation, and from 25° C to 20° C decreased egg accumulation.

(3) Egg accumulation in cultures of solitary queens decreases continuously irrespective of temperature change.

(4) In queen worker colony fragments, the numbers of eggs accumulating are readjusted after 14 days to a value approximating to that before the temperature change.

(5) In *M. laevinodis* there is a corresponding alteration of average egg size after 14 days. Egg size decreases as temperature rises.

(6) No isolated *M. rubra microgyna* queens survived long enough to establish colonies.

(7) There is evidence that the macrogyne may be more suited to act as a colony foundatrix than the microgyne.

(8) 20° C presents a closer approach to optimal conditions for colony foundation than does 25° C.

(9) These results are discussed in relation to mechanisms of caste determination and colony foundation in ants.

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